# SKELETAL MUSCLE AS A PRIVILEGED SITE FOR ORTHOTOPIC SKIN ALLOGRAFTS*

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It is well established that allograft rejection may be delayed or even abrogated in sites devoid of lymphatics, such as the brain (1), the anterior chamber of the eye (2), the hamster's cheek pouch (3, 4), and skin pedicle flaps in guinea pigs and rats (5, 6). Certain other sites, including the testicle (7) and subcutaneous fat (8) may also afford grafts some measure of protection from the normal rigors of transplantation immunity on the basis of diminished though not absent lymphatic circulation. Most of these so-called "immunologically privileged" sites have the practical disadvantage that the alien tissue sustained is inaccessible for repeated visual evaluation or procurement of biopsy specimens. Although not handicapped in this sense, skin pedicle flaps are extremely vulnerable to ischemic destruction, apart from being tedious to prepare and maintain in a viable condition.

Various studies have indicated that skeletal muscle is poorly endowed with lymphatics (9, 10) and a recent pilot experiment indicated that in guinea pigs skin allografts transplanted in "open style" to the panniculus carnosus, stripped of its epimysium, enjoy prolongation of survival and suggested that this bed might have some application as a privileged site (11). The work to be reported extends this finding to rats and evaluates the contributions of lymphatic abnormality, stress, immunologic enhancement, incompatibility at the major (Ag-B) histocompatibility locus, and graft dosage, to the increased allograft survival times.

## *Materials and Methods*

Adult rats of domestically maintained syngeneic Fischer (Ag-$B^1$), Lewis (Ag-$B^1$), and DA (Ag$B^4$) strains were used without regard to sex, with Fischer animals serving as recipients in all experiments.

*Ear Skin Grafts.*—1 cm in diameter, were cut with the aid of a trephine from the pinnae, care being taken to remove the adherent median ear cartilage from the thin sheets of skin.

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Large ear skin grafts comprised the skin removed from both sides of an excised pinna; their aggregate are being 3–4 $cm^2$.

*Grafts Beds.*—These were cut in the close-clipped skin of the lateral thoracic wall under chloral hydrate anesthesia, supplemented with ether as required.

"Fitted grafts" were those placed in beds which extended down to the level of the panniculus carnosus and which were just large enough to receive them (Fig. 1 *a*).

"Open fit grafts" were placed on standard, very extensive beds prepared by sharply incising 3 × 5 cm rectangular outlines in the skin of the lateral thoracic wall, grasping one corner with a hemostat and then tearing the skin as cleanly as possible from the underlying panniculus carnosus muscle. This procedure resulted in the stripping away of the epimysium as well as the overlying fascial connective tissue in which course both blood and lymphatic vessels serving the skin. Skin grafts were placed at the centers of these beds so that their margins were widely separated from host skin (Fig. 1 *b*).

"Skin islands" were 1.5 × 1.5 cm squares of skin left intact at the centers of large open-fit beds. Shallow, circular beds were cut in these islands to receive fitted ear skin allografts (Fig. 1 *e*).

*Dressings.*—They included plaster of Paris impregnated bandage, and were applied around the entire thorax according to our standard procedure (see 12). Primary inspection was usually carried out on the 8th postoperative day and subsequent inspections at 2–3 day intervals. Dressings were reapplied so long as unepithelialized granulation tissue was present.

*Visualization of Afferent Lymphatic Vessels and their Draining Lymph Nodes.*—This was accomplished by intradermal injection, via a no. 30 gauge needle, of a mixture of equal volumes of 2% aqueous solutions of Berlin Blue and Patent Blue V (5).

## EXPERIMENTS AND OBSERVATIONS

*Base-Line Data: Survival Times of Fitted Allografts.*—To provide the necessary controls for the experiments to be described standard, fitted 1 cm diameter ear skin grafts from Lewis and DA donors were transplanted to different panels of Fischer hosts (see Table I, experiments 1 and 2). The median survival time (MST)[1] of Lewis → Fischer grafts was 10.5 ± 1.1 days and that of DA → Fischer grafts was 8.4 ± 0.33 days.

*Fate of Open-Fit Allografts on Extensive Beds.*—The 1 cm diameter skin allografts transplanted in open style were extremely healthy and well-united to their beds by the 8th postoperative day and, with both donor/host strain combinations, the majority significantly outlived their "fitted" controls (Table I, experiments 3 and 4). Grafts that lived long enough became surrounded by annuli of outgrowing hyperplastic epithelium which frequently made contact and fused with ingrowing native epithelium from the wound margins. Although maintenance of the dressings on the wounds retarded contracture (13), eventually this process progressed, apparently eliminating the resurfaced granulation tissue which developed in the wound, to the point where graft dermis and host skin dermis became juxtaposed, usually after about the 20th day. Regeneration of fur took place on many of the long-lived allografts. Rejection of all grafts, when it finally occurred, was an acute rather than a chronic process; total sloughing of the epithelium being complete within 1–2 days of the first indication of its weakness. Compatibility with the host

[1] *Abbreviation used in this paper:* MST, median survival time.

TABLE I

*Survival Times and MST's of Skin Allografts Transplanted to Different Kinds of Beds on Fischer Hosts*

| Exp. no. | Graft donor | Type of graft and bed | No. of hosts tested | Distribution ofallograft survival times | | | | | | | Median survival time |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | <10 | 11–13 | 14–16 | 17–19 | 20–24 | 25–30 | >30 | |
| | | | | | | | | | | | *days* |
| 1 | Lewis | 1 cm ear skin-"fitted" (control) | 10 | 4 | 6 | | | | | | 10.5 ± 1.1; SD* 1.2 |
| 2 | DA | 1 cm ear skin-"fitted" (control) | 22 | 21 | 1 | | | | | | 8.4 ± 0.33; SD 1.12 |
| 3 | Lewis | 1 cm ear skin-"open-fit" | 17 | | 2 | 2 | 4 | 3 | 6 | | 20.8 ± 3.1; SD 1.38 |
| 4 | DA | 1 cm ear skin-"open-fit" | 16 | 3 | 3 | 2 | | 2 | 4 | 2 | 20.5 ± 3.8; SD 1.41 |
| 5 | Lewis | Large ear skin-"open-fit" | 16 | | 1 | | 5 | 6 | 4 | | 20.8 ± 1.5; SD 1.22 |
| 6 | DA | Large ear skin-"open-fit" | 14 | 1 | 4 | 2 | 1 | 6 | | | 18.0 ± 6.4; SD 2.0 |
| 7 | Lewis | 1 cm trunk skin-"open-fit" | 11 | 1 | | 2 | 3 | 1 | 2 | 2 | 20.2 ± 6.5; SD 1.75 |
| 8 | DA | 1 cm trunk skin-"open-fit" | 9 | 3 | 6 | | | | | | 11.9 ± 1.09; SD 1.15 |
| 9 | Lewis | 1 cm ear skin-"fitted on animal with large wound on opposite side (trauma control) | 10 | | 6 | 4 | | | | | 12.2 ± 0.37; SD 1.06 |
| 10 | DA | 1 cm ear skin-eccentric, "open-fit," touching margin | 11 | 4 | 7 | | | | | | 9.3 ± 0.53; SD 1.15 |
| 11 | DA | 1 cm ear skin-eccentric, "open-fit," 1 cm from margin | 11 | 2 | 3 | 3 | | 2 | 1 | | 14.2 ± 3.2; SD 1.41 |
| 12 | Lewis | 1 cm ear skin-skin island | 11 | | 1 | 3 | 3 | | 1 | 3 | 19.8 ± 4.12; SD 1.52 |
| 13 | DA | 1 cm ear skin-skin island | 7 | 2 | 3 | | | 2 | | | 10.8 ± 1.56; SD 1.27 |
| 14 | Lewis | 1 cm ear skin-skin island bed dissected 7–9 days before grafting | 6 | | 2 | 1 | | | 1 | 2 | |

* Standard deviation.

at the Ag-B locus did not appear to be an important factor determining the longevity of the grafts since both the survival time distributions and the MST's of both series of allografts were very similar: Lewis → Fischer; MST 20.8 ± 3.1 days; DA → Fischer; MST 20.5 ± 3.8 days.

*Influence of Dosage on Open-Fit Allograft Survival.*—To find out whether the amount of allogeneic skin transplanted exerted any influence on its longevity on the panniculus, large composite grafts comprising all the skin that could be obtained from both sides of the median ear cartilage of a rat's pinna (3–4

$cm^2$) were transplanted to standard extensive wounds. The size of these grafts necessarily resulted in their perimeters approximating host skin sooner than was the case with the small grafts. Again, this mode of grafting conferred considerable protection upon both Lewis and DA allografts, with the results of the former being as good as those obtained when the smaller grafts were used. However, the performance of the large DA grafts was inferior to that of their smaller counterparts (see Table I, experiments 5 and 6).

When 1 cm diameter grafts of trunk skin, which is much thicker than ear skin, were transplanted to extensive beds, those from Lewis donors fared just as well as ear skin, but those from the DA donors only outlived their controls by a few days (Table I, experiments 7 and 8). No satisfactory explanation can be afforded for this disparity in results.

*Analysis of the Basis of the Prolonged Survival of Skin Allografts on Extensive Panniculer Beds.*—There are three obvious factors which, either singly or in combination, might contribute to the observed impairment of allograft rejection: (*a*) operative stress leading to increased corticosteroid production, which includes hormones with immunosuppressant properties, (*b*) inadequacy of the lymphatic drainage in the graft bed, resulting in attenuation of the afferent pathway of the immunologic reflex, and (*c*) exposure of the host to antigenic material via the venous rather than the lymphatic route, favoring the development of humoral rather than cellular immunity, and so leading to the phenomenon of immunologic enhancement, i.e., the grafts may be capable of "self-enhancement," as in the case of renal and cardiac allografts in rats (14, 15). The experiments now to be described were designed to discriminate between these possibilities.

*Influence of stress:* Standard large wounds were prepared on the right thoracic walls of Fischer hosts but instead of placing 1 cm diameter Lewis test grafts at their centers, these grafts were "fitted" into beds on the contralateral sides of the hosts' thoraxes (Fig. 1 *c*). This resulted in a small but significant prolongation of graft survival, the MST being extended to 12.2 ± 0.37 days (Table I, experiment 9). A second, independent appraisal of the influence of stress was made by placing 1 cm DA ear skin allografts in eccentric locations on extensive beds so that one point on their perimeter was in direct contact with host skin (Fig. 1 *d*). Again, these grafts displayed only a trivial prolongation of survival, (Table I, experiment 10), indicating that stress could have made only a minor contribution to the longevity of the open-fit grafts.

However, of a separate panel of 12 DA skin grafts which were placed eccentrically so that a distance of 1 cm of wound bed separated the graft and wound margins at their nearest point, six displayed very significant prolongation of survival (to 14–25 days: Table I, experiment 11). These observations suggested that intact skin at the wound margins is a more consistent source of "something" essential for graft rejection than the panniculus bed. The most obvious candidate here, of course, is lymphatic drainage (though there are other more

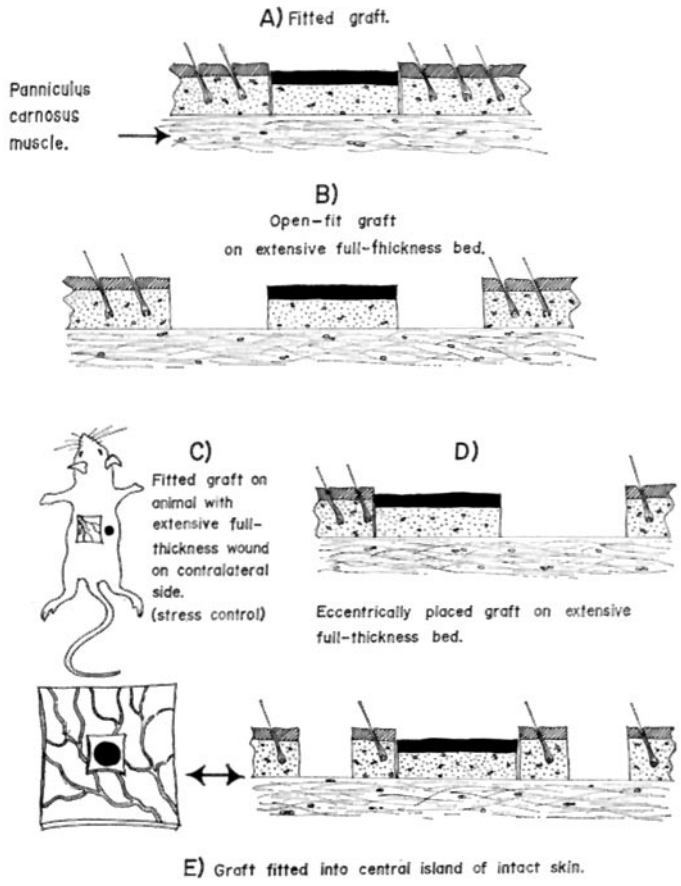


FIG. 1. Illustrating the various types of graft bed bearing ear skin allografts.

remote possibilities, e.g., skin might be richly endowed with antigen-sensitive cells that can recognize transplantation antigens).

To explore this possibility standard 1 cm allografts were fitted into beds cut in the centers of small islands of intact skin left at the centers of extensive muscle beds (Fig. 1 *e*). It was reasoned that if the longevity of the open-fit grafts was due to paucity of their lymphatic drainage, this situation should apply to intraisland beds, but if contact with host skin having an intact blood supply is the essential feature for procuring rejection, then intraisland grafts should display normal susceptibility. Lewis allografts fared extremely well in this site, having an MST of 19.8 $\pm$ 4.12 days, and 3/15 grafts lived longer than 50 days. However the results with DA grafts were disappointing, the MST being 10.8 $\pm$ 4.17 days, and only 2/7 grafts surviving beyond 20 days (Table I, experiments 13 and 14).

*Dye Injection Studies.*—Attempts to study the lymphatic drainage status of the panniculus muscle at various stages after grafting gave equivocal results because of the tendency of the dye to leak and spread rapidly over the surface to the wound edges where it was rapidly taken up by the lymphatics. Injection of the healed-in thin ear skin grafts also proved unsatisfactory since, with surviving grafts, it was difficult to confine the inoculation to graft connective tissue and with rejecting grafts the vessels had lost their patency. However the "full-thickness" skin islands proved ideal sites for dye injection, especially when they were 4 days post preparation. At and beyond this time their initially raw dermal margins had become reepithelialized, preventing lateral leakage of dye from transected lymphatics. It seemed reasonable to assume that the lymphatic drainage status of these islands would be representative of that of the healed-in, open-fit skin grafts.

Dye injected into skin islands of less than 11 days' standing gave no evidence of entering regional lymphatics or of reaching the draining axillary and brachial nodes (Table II). However dye did escape into lymphatics and enter the nodes from 8/11 islands injected 18 or more days after their preparation. When islands of more than 22 days' standing were injected dye was transmitted to the regional nodes in 5/5 tests. This result was not unexpected since contracture had resulted in the close approximation of graft skin with host skin.

On the basis of these findings, it was predicted that if lymphatics are important in graft rejection, allografts inlaid into skin islands prepared 7–9 days previously should also enjoy some deferment of rejection. This prediction was fulfilled by the finding that 3/6 delayed Lewis intraisland grafts survived for longer than 25 days (Table I, experiment 14).

TABLE II

*Results of Injecting Dye into Intact Skin Islands at Centers of Extensive Full-Thickness Wounds, at Various Times after Their Preparation*

| Day of injection after preparation of "skin" island | Whether regional axillary node stained (+ or −)* | |
|---|---|---|
| 5 | −− | 2/11 nodes stained at 5–15 days. |
| 9 | −− | |
| 11 | −−−±‡+ | |
| 15 | −+ | |
| 18 | ++ | 8/11 notes stained at 18–30 days |
| 21 | −− | |
| 22 | −+ | |
| 26 | ++ | |
| 30 | +++ | |

* Each score relates to an independent test conducted on a single rat.

‡ The regional node was not stained in this animal but a dye-stained lymphatic-like vessel was observed passing directly through the chest wall from beneath the skin island.

*Influence of Immunologic Enhancement.*—To determine the extent to which the phenomenon of immunologic enhancement might have participated in weakening the host's reactivity to skin allografts, Fischer rats which had previously manifested attenuated reactivity to open-fit allografts were rechallenged on the opposite side of their trunks with "fitted" allografts from the original donor strain. In all instances these animals behaved as if they had been sensitized.

In another series of tests primary open-fit and fitted Lewis grafts were excised from two groups of five Fischer rats after they had been in residence for 4 days. The animals were then challenged with secondary fitted Lewis grafts on the opposite side of the thorax. The results (Table III, experiments 1 and 2) indicate that wereas 4 days' residence of a primary fitted graft lead to sensitization, a similar period of residence of an open-fit graft had no influence on the subsequent reactivity of the host.

In an extension of this experiment surviving primary open-fit DA skin grafts on Fischer hosts were excised 8–20 days after transplantation and the animals rechallenged with open-fit grafts of DA skin, to determine the influence of the primary grafts on the reactivity of the host (Table III, experiment 3). The results indicate that residence of the primary graft for as long as 20 days was insufficient to sensitize all hosts, though in the majority of cases 9–10

TABLE III

*Summary of "Second-Set" Grafting Experiments to Determine Whether Residence of the Primary "Open-Fit" Allograft Had Significantly Altered Host Reactivity*

| Experiment | Donor strain | Time first graft in residence | No. of tests | Survival time of 2nd grafts |
|---|---|---|---|---|
| | | *days* | | *days* |
| 1. Excision of surviving primary, open-fit graft followed by transplantation of "fitted" allograft on contralateral side | Lewis | 4 | 5 | 3 × 11, 13, 14 |
| 2. Excision of primary "fitted" graft followed by transplantation of second "fitted" graft on contralateral side | Lewis | 4 | 5 | 3 × 8, 2 × 9 |
| 3. Excision of primary open-fit allograft followed by transplantation of open-fit allograft on contralateral side | DA | 20 | 3 | 8, 11, 19 |
| | | 15 | 1 | <8 |
| | | 10 | 5 | <7, 8, 2 × 9, 11 |
| | | 9 | 14 | <7, 5 × 8, 6 × 11, 15, 20 |
| | | 8 | 10 | 2 × 10, 5 × 11, 13, 15, 24 |
| 4. Transplant fitted graft to animal bearing open-fit graft of 7 day's standing | DA | — | 14 | *15/12; 6 × 5/>8; 14/>8<br>3 × <14/<8; 3 × 12/<8 |

* Results expressed as survival times of primary grafts/survival times of second grafts.

days' exposure to the primary graft was sufficient to initiate weak sensitization.

Finally, a panel of 14 Fischer rats which had received open-fit DA grafts on the right thoracic wall 7 days previously received fitted grafts on the left side. None of these second-set grafts showed prolonged survival (see Table III, experiment 4). The observation that none of the primary grafts lived longer than 15 days, and most of them were destroyed concomitantly with their accompanying second-set grafts, suggested that the latter compromised the survival of their predecessors. This, again, indicates that central inhibition of response was not involved in the prolongation of graft survival observed.

## DISCUSSION

The present findings confirm and extend to the rat an observation previously made in the guinea pig (11), that skin allografts transplanted in open style to extensive beds afforded by bare panniculus carnosus muscle enjoy a highly significant prolongation of survival—by a factor of about 2—provided that the graft margins do not make contact with host skin.

That this abrogation of allograft reactivity was not due to operative stress, and an associated release of corticosteroid hormones, or to some kind of induced "central" weakening of response, such as immunological tolerance or enhancement, was established on the basis of several discriminating experiments. For example: (*a*) only feeble prolongations of survival resulted when the grafts were fitted into small beds on one side of the hosts' trunks and extensive panniculus wound beds were prepared on the contralateral sides or when the grafts were placed marginally in contact with host skin on the extensive beds, and (*b*) no prolongation of survival was enjoyed by secondary fitted skin allografts transplanted to hosts bearing healthy, open-fit grafts of 7 days' standing.

Interference with the afferent limb of the immunologic reflex is a much more likely explanation, especially since; (*a*) allograft survival was also prolonged by fitting them into small beds prepared in intact residual islands of skin at the centers of extensive areas of raw panniculus and (*b*) dye injection studies on such skin "islands" at various times after their preparation indicated a transient deficiency of lymphatic drainage from the wound beds to the regional nodes persisting until about the 15th postoperative day.

It is necessary to emphasize that the present findings are easily reconciled with the familiar observation that skin allografts placed centrally upon large beds afforded by the panniculus carnosus in the rabbit are promptly rejected (16). In this species there is a natural cleavage plane between the dermis and the panniculus so that the epimysium and its rich vascular and lymphatic network are normally left intact when such extensive beds are prepared. In rats and guinea pigs, by contrast, this cleavage plane is absent and the mode

of preparation of these beds results in either the removal *in toto* or at least heavy damage to the epimysium and its associated vasculature.

The present observations are consonant with the general thesis that the various known immunologically privileged sites which sustain vascularized skin allografts do so by virtue of the absence or considerable impairment of lymphatic drainage, i.e. a deficiency in the afferent pathway of the immunologic reflex. Precisely what function lymphatic channels fulfill in mediating the rejection of free tissue allografts remains to be determined. The conventional view is that they transmit antigenic material to the lymph node. Alternatives are that they transmit host lymphocytes which have been "primed," i.e. have fulfilled the act of antigenic "recognition," peripherally (17–19). Another possibility which is not mutually exclusive is that they transmit leukocytic "passenger" cells from the graft which function as antigen when they have percolated into the draining node (20, 21).

Apart from their possible usefulness in sustaining allografts in nonimmunosuppressed hosts—whether for experimental or for clinical purposes—and the light they have shed on the pathophysiology of allograft rejection, the existence of immunologically privileged sites has some important theoretical and clinical implications: (*a*) It challenges the thesis that normal, unsensitized animals already possess a significant proportion of lymphocytes endowed with the capacity to react against major locus-incompatible allogeneic cellular antigens, i.e. the only difference between a normal animal and a sensitized animal is that the latter has a higher incidence of such cells (22).

(*b*) If the postulated and much discussed cell-mediated immunological tumor surveillance mechanism (23) really exists, one would have anticipated that natural privileged sites in which rapidly proliferating populations of epithelial or other cells are present—as in the cornea or the hamster's cheek pouch—would be common sites for the development of malignancies. There is no evidence that this is so. However, of possible relevance is the high incidence of reticulum sarcomas in the brains of immunosuppressed renal transplant patients (24). It has been suggested that the brain's alymphatic status, in conjunction with the immunosuppression results in a blunting of immunologic recognition and the initiation of cellular immunity directed against the tumor specific antigens at an early stage. It is also tempting to relate the relatively high incidence of tumors which develop in cutaneous burn scars in man, noted by Celsus in the first century (25), to the immunologically privileged status which healed burn lesions have been shown to possess (26).

## SUMMARY

A semi-privileged status for rat skin allografts may be achieved by placing them on extensive open beds formed by panniculus carnosus muscle which prevents contact of the transplant with host skin. Such allografts enjoy ap-

proximately a twofold increase in their life expectancy, even if transplanted across a strong histocompatibility barrier. Experiments are described which rule out stress or a "central" weakening of response, such as enhancement, as explanations of this phenomenon.

Intact skin "islands" separated from surrounding host skin on all sides by a broad border of bared panniculus were also found to serve as privileged sites. Dye injected into these islands failed to reach the regional nodes until about the 15th day after their preparation. These studies indicate that a lymphatic deficit is responsible for the observed privileged status of the allografts.

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