TRANSPLANTATION IMMUNITY IN BONE HOMOGRAFTING*

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The knowledge that homografts of many tissues have a limited period of survival, and the revival of the theory of tissue immunity (Gibson and Medawar 1943, Medawar 1944, 1945) makes it of interest to know how bone homografts compare with other tissues in this respect.

Since 1878 homografts of bone have been known to be clinically effective (Macewen 1912), and the introduction of the bone bank (Inclan 1942) has led to a great increase in their use. The role of tissue immunity reactions in bone homografting has received little attention until recently, and reliable information concerning cell survival and the source of bone formation in these clinically effective grafts is lacking.

The present study was undertaken to determine whether homografts of bone behaved in the same way as those of other tissues with respect to cell survival and the production of transplantation immunity, and to consider the importance of such a reaction if found to be present.

METHOD AND MATERIALS

It was decided to make a detailed histological comparison between autografts and homografts of bone, and also to study bone homografts in animals which had been "immunised" by means of a prior skin homograft from the same donor. Bone homografts which had been freeze-dried were to be compared with fresh material by a study of their histological progress: in addition to this the rejection times of subsequent skin homografts from the same donors were to be determined, the purpose being to demonstrate whether freeze-drying influences the behaviour of a bone homograft, or the reaction of the host tissues to it.

The experiments were carried out on rats 60 to 100 days old. Wistar albino and hooded strains were used so that homografts could be exchanged between strains: it was hoped in this way to avoid any "matching" of tissue antigens in host and donor.

The bone grafts were obtained from the proximal half of the ilium and had an average weight of 38 milligrams. After all soft tissue had been scraped off they were chipped into fragments about a millimetre long. The graft was placed in a slit made in the lumbar paravertebral muscles. An extra-skeletal site was used so that the progress of the graft would not be complicated by the inclusion of skeletal tissue from the host.

Freeze-dried bone was prepared by continuous evacuation over phosphorus pentoxide at —30 degrees centigrade for two weeks. This achieved drying to 1 per cent of the original water content.

Unless otherwise stated thirty-two rats were used in each series. Two rats were killed every second day until the eighteenth day, then at four, six and eight weeks and thereafter at monthly intervals to six months (in a few instances to one year). The number of animals at a particular period was sometimes increased when confirmation of some specific point was required, but the figures quoted in the text apply only to the numbers in the original series.

When the animals were killed a block of muscle containing the bone graft was excised

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Rejection times of first set skin homografts in rats.

Rejection times of second set skin homografts in rats.
and fixed in formalin. Paraffin sections were prepared after decalcification with neutral E.D.T.A.* and were stained with haematoxylin and eosin.

Skin grafts were carried out with minor modifications of the technique described for "fitted grafts" by Billingham and Medawar (1951) and Woodruff and Simpson (1955). A circular piece of skin about a centimetre in diameter was dissected off the panniculus carnosus of the lateral chest wall and sutured to a defect created by the removal of a similar graft on another rat. The graft was protected by a dressing of vaseline gauze and a plaster-of-Paris jacket.

A series of skin homografts was carried out to establish the rejection time using this technique. A mean survival time of 10.9 days was found in ninety-three grafts† (Fig. 1).

TABLE I
SERIES OF EXPERIMENTS

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<table>
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<tr>
<td>1.</td>
<td>Bone autograft</td>
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<td>2.</td>
<td>Bone homograft in normal rats</td>
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| 3. | (a) Bone homograft three weeks after skin homograft from the same donor  
(b) Bone homograft four months after skin homograft from the same donor |
| 4. | Bone homograft four weeks after bone homograft from the same donor |
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| 7. | Skin homograft three weeks after fresh bone homograft from the same donor |
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Second skin grafts to rats immunised fifteen to nineteen days before by skin homograft from the same donor underwent an earlier rejection, a mean survival time of 6.3 days being found with fifteen grafts (Fig. 2). Table I lists the series of grafts that were studied.

RESULTS

Series 1: Bone autograft—Apart from a few osteocytes which survived in some of the grafts, almost all the grafted bone died. This was evident from empty lacunae and intensely basophilic staining of any residual osteocyte nuclei. Organisation of the haematoma was complete in six to eight days.

At four days new woven bone began to appear on the surface of the graft fragments. This new bone grew vigorously, linking the graft fragments and extending into the surrounding connective tissue (Figs. 3 to 5). At fourteen days newly formed lamellar bone was seen for the first time; at sixteen days the new bone which had hitherto been arranged in a haphazard manner began to show organisation in irregular spheres or ossicles (Fig. 6). These structures were occupied by haemopoietic marrow from the beginning—even before their walls were complete—and they became progressively more regular in outline. They were elongated, their long axes corresponding with the direction of the adjacent muscle fibres (Figs. 7 and 8).

* Ethylene diamine tetra-acetic acid. The decalcifying solution is prepared by dissolving 250 grammes of the disodium salt of E.D.T.A. in 1,750 millilitres of distilled water, and adjusting the pH to 7.0 by the addition of sodium hydroxide.

† Recorded rejection times for first set skin homografts in rats by other workers: 8 days, Levinson and Necheles (1956); 8.1 days, Lehrfeld and Taylor (1953); 11.6 days, Rabinovici (1947); 13 days, Stark (1951); 13 to 14 days, Woodruff and Simpson (1955).
Nodules of cartilage developed in six of thirty-two graft sites (Fig. 9) and in one instance became incorporated in the wall of the ossicle. Osteoclastic resorption proceeded from the fourth day, removing dead graft and new bone which did not contribute to the ossicle. After the mature ossicle had formed little further osteoclast activity was seen. The dead tissue of the original graft was not always completely removed, being occasionally found incorporated in the wall of the ossicle (Fig. 8). The reaction of the host site to the graft consisted initially of rapid invasion of the haematoma by granulation tissue and round cells. This settled by eight days to a less vascular fibrous tissue which finally became a thin fibrous periosteum to the forming ossicle. All grafts in this series conformed to this pattern.

Series 2: Bone homograft in normal rats—Initially the findings were very much the same as those described for autografts in Series 1. Only a few osteocytes could be identified in the grafted bone. Granulation tissue rapidly extended through the graft area. New bone appeared on the graft surface at four to six days and grew until eight days (Fig. 10). In contrast to the autograft, this new bone formation, although variable in amount, was usually scanty: in three of fourteen grafts of six to eighteen days' duration it could not be identified.

After eight days the progress was entirely different from that of the autograft. The surface layer of osteoblasts on the new bone became flattened and more basophilic and quickly disappeared (Figs. 11 and 12). The deeper osteocytes of the new formed bone also became pyknotic and fragmented (Figs. 12 and 13) and in their turn, disappeared. In those grafts in which a large amount of new bone was produced, the dissolution of the bone cells was frequently delayed until sixteen or even eighteen days (Fig. 14). Death of this new bone left a characteristic vacuolated bone matrix, the empty lacunae of the immature bone being large in comparison with the amount of matrix. This enabled the early phase of new bone

**FIG. 3**
Autograft, six days (× 315). The lacunae of the graft are empty, or contain only shrunken remnants of osteocytes. New bone has begun to grow on the surface of the graft and is extending into the surrounding connective tissue.
FIG. 4
Autograft, ten days (×315). The original graft is largely acellular. There is vigorous proliferation of new bone from the graft surface.

FIG. 5
Autograft, fourteen days (×105). Growth of new bone continues. Its arrangement appears quite haphazard.
Autograft, eighteen days (×105). The bone has now grown into an irregular sphere containing haemopoietic marrow. Note the active remodelling in progress as shown by the osteoblastic activity on some surfaces and osteoclastic activity on others.

Autograft, six weeks (×105). The ossicle has become complete and more regular. It is elongated in the direction of the adjacent muscle fibres.
Fig. 8
Autograft, six months (×75). A late ossicle. A few small fragments of original graft are incorporated in the cortex of the ossicle. Note the complete absence of reaction in the surrounding tissue.

Fig. 9
Autograft, twelve days (×315). Showing area of cartilage formation at the graft site.
FIG. 10
Homograft, six days (×315). Showing new bone formation on surface of graft with osteoblast border in part. Most of the lacunae in the graft are empty.

FIG. 11
Homograft, eight days (×290). The surface layer of osteoblasts on the new bone are flattened and more basophilic than normal, while the cells in the newly formed bone still appear normal. Contrast this with the autograft at ten days (Fig. 4) in which the surface osteoblasts retain their cuboidal structure.
FIG. 12
Homograft, ten days (×315). Showing disappearance of surface osteoblasts and pyknosis of the cells in the new bone; osteoclastic resorption of the original graft and of the new bone is proceeding.

FIG. 13
Homograft, twelve days (×315). Some of the lacunae of the new bone are now empty while the remaining cells show degenerative changes. The inflammatory reaction to the graft is well shown.

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FIG. 14
Homograft, sixteen days (×315). This photomicrograph illustrates the fact that when a large amount of new bone is formed in relation to a homograft many lacunae still contain bone cells at sixteen days.

FIG. 15
Homograft, four weeks (×315). The new bone which has formed in relation to the bone homograft, although dead and acellular, is still identifiable because of its characteristic open matrix.
Homograft, five months ($\times 315$). Active new bone formation of the late phase has commenced on the graft surface. The inflammatory reaction to the homograft is still present.

Homograft, ten weeks ($\times 315$). Another example of the late phase of homograft new bone. Note that it remains closely applied to the surface of the original graft from which it is separated by a clear cement line. This new bone is lamellar in character. The inflammatory reaction is still present in the surrounding connective tissues.
formation to be identified even after the death of the cells concerned (Fig. 15). Resorption of this dead new bone proceeded rapidly, so that after four weeks it could be identified in only three of fourteen grafts.

### TABLE II

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<tr>
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<th>Autograft</th>
<th>Homograft</th>
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<tr>
<td></td>
<td>Early phase</td>
<td>Late phase</td>
</tr>
<tr>
<td>Duration of growth and survival</td>
<td>Four days to at least six months</td>
<td>Four weeks to at least six months</td>
</tr>
<tr>
<td>Type of bone</td>
<td>Woven at first, becoming lamellar at fourteen days</td>
<td>Woven</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lamellar</td>
</tr>
<tr>
<td>Quantity</td>
<td>Profuse</td>
<td>Variable but usually scanty</td>
</tr>
<tr>
<td></td>
<td>100 per cent</td>
<td>78 per cent</td>
</tr>
<tr>
<td>Incidence</td>
<td></td>
<td>30 per cent</td>
</tr>
<tr>
<td>Progress</td>
<td>Forms ossicle containing haemopoietic marrow</td>
<td>Resorbed rapidly after death</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forms plaque on surface of dead graft</td>
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A second phase of new bone formation sometimes occurred in homografts of four weeks and older. This phase consisted of slowly growing lamellar bone. It was never large in amount, and did not organise into an ossicle. It remained closely applied to the surface of the graft, a well marked cement line being present at the interface (Figs. 16 and 17). This type of new bone was seen in five of fourteen grafts of four weeks and older.

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General curves indicating the amount and duration of living new bone in autograft (A), homograft early phase (B) and homograft late phase (C).
Freeze-dried homograft, twelve days (×105). The graft bone is entirely acellular. No new bone has developed. The inflammatory reaction is less intense than that found in relation to the fresh homograft at this stage (cf. Fig. 13).

Freeze-dried homograft, six months (×315). Lamellar new bone growing on the surface of a long term freeze-dried homograft.
Table II lists the characteristic features of the autograft new bone and the early and late phases of homograft new bone.

Figure 18 is a graphic impression of the time relationship of these three types of bone and their relative quantity.

Osteoclasts were present from the fourth day, but resorption of the dead homograft proceeded more slowly than the dead autograft. The local response of the host tissues resembled that to the autograft until the eighth day. Thereafter there was an increase in fibrous tissue and a heavy infiltration with lymphocytes and varying numbers of plasma cells, eosinophils and histiocytes. This inflammatory reaction persisted until four to five months.

Series 3a: Bone homograft three weeks after skin homograft from the same donor—In this series, as in the previous ones, only very few osteocytes survived transplantation. Neither the early nor the late phase of homograft new bone formation occurred. Resorption of the graft proceeded slowly, as in Series 2. The inflammatory reaction of the host tissues to the graft was similar to that in Series 2, but more intense in the early stages.
Series 3b: Bone homograft four months after skin homograft from the same donor—There were six animals in this series. The bone grafts were examined at thirteen days. In each case new bone characteristic of the early phase of homograft new bone was present. There was an inflammatory reaction to the grafts, as in Series 2.

Series 4: Bone homograft four weeks after bone homograft from the same donor—In this group there were two grafts to examine in each animal, the second grafts being of two days' to six months' duration whereas the first grafts were twenty-eight days older. Occasional osteocyte survival was observed in both. The second grafts gave two examples of early type new bone in the fourteen grafts from six to eighteen days. Late phase new bone occurred in three of fourteen grafts of four weeks' duration and older. In the first grafts, the late phase new bone occurred in six of thirty-two grafts. The inflammatory reaction and slow rate of resorption of these grafts corresponded to Series 2.

Series 5: Freeze-dried bone homografts—No survival of osteocytes was observed in this series, all lacunae in the grafted bone being empty. No new bone occurred. Giant cells resembling osteoclasts were present from the sixth day and resorption proceeded slowly. The inflammatory reaction was less intense and later in onset than with the fresh homograft (Fig. 19). An additional number of longer-term grafts, of five to ten months' duration, was added to the
original series. Of these, three of fourteen showed small amounts of new bone with the characteristics of late phase homograft new bone (Fig. 20).

**Series 6: Freeze-dried bone homograft four weeks after skin homograft from the same donor**—This series behaved as Series 5. No survival of osteocytes was seen. No new bone developed by six months. Resorption associated with the presence of osteoclast-like giant cells progressed slowly. A mild inflammatory reaction occurred, as in Series 5.

**Series 7: Skin homograft three weeks after fresh bone homograft from the same donor**—There were fourteen animals in this series. The skin grafts had a mean survival time of 5-6 days (Fig. 21).

**Series 8: Skin homograft three weeks after freeze-dried bone homograft from the same donor**—There were fourteen rats in this series. The skin grafts had a mean survival time of 12-0 days (Fig. 22).

Figure 23 summarises the incidence of the early and late types of homograft new bone in the different series.

**DISCUSSION**

When these results are considered together certain patterns of behaviour of autograft and homograft bone emerge.

Survival of a few osteocytes was observed in all series in which fresh bone, whether autograft or homograft, was used, but not in freeze-dried bone grafts. Autograft gave rise to new bone which became organised into an ossicle with haemopoietic marrow. All bone, living or dead, which did not contribute to the ossicle was quickly removed. There was no inflammatory reaction to the autograft.

Fresh homograft was associated with two separate and distinct phases of new bone formation, early and late, the early phase terminating in death of the new bone by eighteen days. The incidence of both these phases was reduced by a previous homograft of bone from the same donor and abolished by a previous homograft of skin from the same donor. Freeze-drying the bone homograft prevented the development of the early phase, but the late phase developed in a few instances after a lapse of many months.

Resorption of the homografts was slower than that of the autografts. Bone homografts provoked a local inflammatory reaction which was more intense with the fresh than with the freeze-dried bone. The survival time of skin homografts was shortened by a previous homograft of fresh bone from the same donor, but was slightly prolonged by a previous freeze-dried bone homograft.

Some investigators have been reluctant to accept the theory of transplantation immunity because of the failure of many attempts to isolate antigens from tissue extracts and because of the failure to demonstrate the presence of antibody in vitro by the classical techniques such as cell agglutination and complement fixation. Billingham, Brent and Medawar (1956a) recently succeeded in isolating the antigens responsible for the homograft reaction in the nuclear fraction of spleen and kidney cells in mice and found it to be a deoxyribonuclear protein. The antigen was very unstable and was destroyed by drying from the frozen state, and by repeated freezing and thawing. They also described a second type of antigen present in the cytoplasm which is more stable and which can be demonstrated by orthodox serological techniques. This antigen does not evoke homograft response, but by some means not yet fully understood it delays the rejection of a subsequent homograft from the same donor—the “enhancement effect.” Bonfiglio, Jeter and Smith (1955) succeeded in demonstrating antigens in extracts of rabbit bone. These may have been of the second category, for injections of this material reduced the reaction to subsequent bone homografts. Curtiss and Herndon (1956) were unable to show that bone possessed antigens of the blood groups in dogs.

There is much evidence that the antibody responsible for bone homograft rejection is bound to mobile cells of the host, and that actual contact of these cells with the cells of the graft is necessary before the reaction can take place. Transplantation immunity can be
transferred to another animal by implants of lymphatic tissue from an immune animal, but not by serum (Mitchison 1954). If the graft is excluded from the cells of the host by means of a diffusion chamber then it does not undergo rejection (Algire, Weaver and Prehn 1954). This may be the explanation for the continued survival of homografts of cartilage and cornea so long as they remain avascular, for in these tissues the cells are protected by a continuous mucopolysaccharide matrix (Loeb 1930, Peer 1954).

Against this background it is possible to draw a number of conclusions concerning the influence of transplantation immunity on bone homografts from the results of the experiments described above.

The observation that skin homografts after a previous bone homograft from the same donor (Series 7) undergo an early rejection, establishes that bone contains tissue antigens which evoke an immune response in the host. It is evidence, too, that bone and skin have antigens in common.

The existence of an immune response to homograft bone allows a clear understanding of the nature of the early new bone formation that occurs in relation to the autograft (Series 1) and the living homograft (Series 2). A latent period before the rejection of homografts of skin and other tissues exists because several days must elapse before the immune state of the host is developed sufficiently to bring about the death of the graft (Medawar 1954). During such a latent period, any surviving osteogenic cells of a bone homograft would have an opportunity of proliferating and forming bone. If, however, bone possesses tissue antigens that can evoke a reaction in the host, one would expect any new bone formed from homograft cells to have a limited period of survival. This is precisely the behaviour of new bone from homografts in the early phase, and is evidence that its origin is from grafted cells and not from the host tissues.

Danis (1956), who observed a similar phase of new bone formation with homografts of bone marrow in rabbits, came to the same conclusion; but Hutchison (1952), who was possibly the first to describe the early phase of homograft new bone, considered it to have arisen from host tissues. He did not, however, give his reasons for this opinion.

The failure of the early phase of homograft new bone to develop in Series 3a in which the animals had been immunised beforehand by a skin homograft is further evidence that this phase arises from cells of the graft, for once transplantation immunity has been established the animal has a heightened resistance to further grafts from the same donor.

The limited duration of transplantation immunity is shown by Series 3b in which each of the bone homografts carried out four months after a skin homograft from the same donor produced early new bone. This is in keeping with the duration of immunity against other tissues in rats recorded by Lehrfeld, Taylor and Converse (1955) and in mice by Billingham, Brent and Medawar (1954).

When the pre-immunising homograft was bone (Series 4) the suppression of the early phase of new bone growth was not so consistent as in Series 3a, for two of fourteen grafts showed new bone of this type. The reason for this may be that muscle is a less satisfactory site for the production of immunity than skin. It is known that levels of immunity may vary with the site of implantation (Medawar 1948; Billingham, Brent and Medawar 1956b; Billingham, Brent and Mitchison 1957).

Freeze-drying appears to inactivate histocompatibility antigens in bone homografts since subsequent skin grafts from the same donor did not undergo an early rejection but survived slightly longer than normal (Series 8). This prolongation of survival time, although small, was statistically significant* (Fig. 22). This may be an example of the "enhancement effect."

* Mean survival time of first set skin homografts—10-9 days. Standard error 0-13. Mean survival time of skin homografts following freeze-dried bone homografts—12-0 days. Standard error 0-29. Difference of mean of the two groups—1-1 days. Standard error of the difference 0-32. Application of the "t" test indicates that this difference is significant at the 0-01 level.
The modified antigenicity of freeze-dried bone homografts is reflected in the reduced inflammatory reaction at the host site. Pre-immunisation of the host did not increase this reaction (Series 6) as it does with some other killed tissue homografts (Darcy 1955).

The continued survival of a few osteocytes within the grafts of fresh homogenous bone despite the demonstrable existence of transplantation immunity is readily explained. Osteocytes are surrounded by bone matrix and are nourished by tissue fluid diffusing through small canaliculi; in an intact graft they do not come in contact with the host cells. Bone is in fact a natural example of Algire et al.'s (1954) diffusion chamber; like it, it excludes host cells and so protects its own cells from the immune reaction.

The bone cells of the early phase of homograft new bone, although often surviving longer than the surface osteoblasts, all die by the eighteenth day. Perhaps the immature matrix is an incomplete barrier to the host cells, or perhaps there is a local interruption of blood supply such as has been so well described by Taylor and Lehrfeld (1953) in the case of the rejecting skin homograft.

The histogenesis of the late phase of homograft new bone remains unsolved. There are two possible sources: from the graft itself or from the adjacent connective tissue of the host which has undergone metaplasia into bone-forming tissue. The fact that transplantation immunity has been established against the graft does not eliminate the possibility that graft cells may be responsible, for it has been suggested that homografted tissues which are kept alive beyond a certain critical period are no longer susceptible to the immune response (Woodruff 1952). One could argue that the living osteocytes which were demonstrable in the fresh homografts might become liberated during the process of resorption and resume osteogenic function. Evidence in favour of the metaplastic origin of this bone is provided by the fact that it developed in relation to several of the freeze-dried bone grafts (Series 5). Freeze-drying kills mammalian skin (Billingham and Medawar 1952) and mature bone (Ray et al. 1952, Pate 1954): in the present experiments there was no histological evidence of cell survival in any of the freeze-dried bone grafts used. Whether bone can in fact induce metaplastic bone formation in adjacent connective tissue is the subject of much discussion at the present time. There are many reports of new bone developing around grafts of dead bone in soft tissues (Wilson 1951, Axhausen 1953, Urist and McLean 1952, Curtiss and Wilson 1953, Maatz 1955, De Bruyn and Kabisch 1955) but others have failed to confirm this (Ray et al. 1952, Ham and Gordon 1952, Keith 1934, Campbell et al. 1953, Danis 1956). The experiments recorded in this paper suggest that dead bone can induce metaplastic bone formation in non-skeletal connective tissue, but that this occurs infrequently and only after a long period.

From this study it is possible to draw the following conclusions which have a bearing on clinical bone grafting.

1. Bone homografts, whether living or dead, are not able to make a significant contribution of living bone tissue (except in a few special instances such as a graft to an identical twin).

2. There is then no advantage of living homograft over preserved material. Indeed, preserved bone may be preferable in view of its altered antigenicity.

3. The lack of a supply of living cells in the homograft, together with its slower resorption, could account for the differences of behaviour that have been observed clinically between autografts and homografts.

**SUMMARY**

1. Fresh bone autografts to a muscle bed in the rat gave rise to vigorous new bone formation from about the fourth day. The graft took the form of a hollow ossicle with central bone marrow at eighteen days: it became progressively more regular in outline and was still present at six months.

2. Fresh bone homografts produced two separate phases of new bone formation—early and late. In the early phase non-lamellar woven bone appeared at about the fourth day, continued
to grow until eight days, and subsequently died. It arose from osteogenic cells of the homograft. In the late phase, which developed in relation to a few grafts after four weeks, the new bone was lamellar in character, and remained closely applied to the graft surface. Evidence is presented that this bone arose by metaplasia of the host connective tissues at the graft site. There was a local inflammatory response to the bone homograft.

3. Both phases of homograft new bone formation were abolished if the animal was prepared by a skin homograft from the same donor four weeks before, but not if four months elapsed between the two grafting procedures.

4. Freeze-dried bone homografts did not give rise to the early phase of homograft new bone but produced a few examples of the late phase after five months. The inflammatory response was less intense with freeze-dried homografts than with fresh homografts.

5. Skin homografts three weeks after fresh bone homografts from the same donor underwent an early rejection at five to six days.

6. Skin homografts three weeks after freeze-dried bone homografts from the same donor had a mean survival time of twelve days, which was significantly longer than the mean survival time of 10-9 days in normal rats.

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