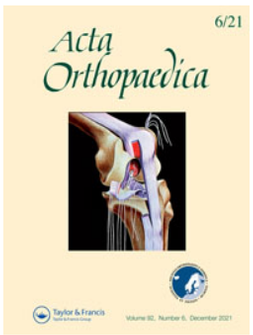

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THE PATTERN OF NEW BONE FORMATION IN ISOGRAFTS OF BONE

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After transplantation of autografts, or isografts, of fresh bone, new bone formation soon occurs. The origin of this new bone is the subject of debate so that although some investigators will concede that it may to some extent be derived from osteogenic cells of the graft itself (Urist & McLean 1951, Ham & Gordon 1952, Hutchinson 1952, Ray & Sabet 1963, Puranen 1966, Bohr et al. 1968), others claim that it is entirely derived by metaplasia of host cells (Barth 1893, Reynolds & Oliver 1949, De Bruyn & Kabisch 1955). Chalmers (1959) has suggested that in the case of some bone homografts there are two phases of osteogenesis: an early phase in which the graft cells participate and which is cut short due to an immune response, and a late phase due to the activity of host derived cells. In the case of isografts, however, no such distinction can be made using histological techniques.

With the development of an objective radiochemical assay of osteogenesis it is possible to make a more dynamic assessment of the pattern of osteogenesis in bone grafts (Elves 1974). Using this approach it has been possible to show that there is evidence of two phases of osteogenesis in isografts of bone, that the early phase has a major graft contribution and that the second phase is host derived.

MATERIALS AND METHODS

Animals used as recipients in this study were 2- to 3-month-old (200–250 g) male rats of the AS inbred strain. Grafts were of iliac bone containing bone marrow also from rats of the AS strain. The attached muscle and the periosteum were scraped off before grafting. Preserved grafts used in this study were frozen AS bone which had been stored at –20° C for 1–2 weeks, freeze-dried AS bone or AS bone which had been decalcified in HCl and then freeze-dried. In some experiments fresh grafts were taken from donor AS strain rats which had been exposed to 650 or 850 rad x-irradia-

tion 24 hours earlier, whilst in other experiments fresh isografts were exposed to 1200 rad x-rays *in vitro* immediately prior to grafting. In order to assess the contribution of the graft marrow to osteogenesis marrow was removed from some fresh grafts by subjecting them to a stream of saline until they became white.

All grafts were placed into a suprapannicular pocket made in the skin of the dorsal surface of the recipient's thorax. Each rat received a fresh intact graft in addition to any of the preserved or treated grafts being examined. Grafts were examined after 1, 2, 3, 4, 6 and 8 weeks.

Osteogenesis was measured at each time interval using Strontium^{85} as previously described (Elves 1974). Strontium^{85} chloride was injected into rats by the intraperitoneal route 3½ days before killing. Results are expressed as an osteogenic index which relates isotope uptake in the graft to that in the cancellous skeleton of the host. It must be emphasised that this technique gives an assessment of osteogenesis over the short period of exposure to isotope, and hence allows a distinction to be made between recent new bone formation and that which has occurred earlier (Elves 1974), a distinction which it is not possible to make using histological techniques. All grafts were also examined histologically after measurement of their contained radioactivity.

RESULTS

Osteogenesis in fresh isografts

One hundred and sixty-three recipients of fresh, marrow-containing isografts have been studied. Between three and five experiments were carried out at each time interval and five or six animals were examined in each experiment. The results are summarised in Figure 1. From these results it is clear that the osteogenesis in most grafts (30/37) examined 2 weeks after grafting was in excess of that in the host's pelvis. The level of osteogenesis rose to a peak at 3 weeks by which time all grafts had an index of more than 1.0. A significant decline then followed. The mean index of osteogenesis at 3 weeks was significantly elevated compared with that at 2 weeks ($P = 0.01$–0.001). Osteogenesis seemed to reach a plateau between 4 and 6 weeks after grafting as the index was not significantly different between these points ($P = 0.7$). Six grafts out of 23 in the rats examined after 4 weeks, and two out of 28 grafts examined after 6 weeks gave indices which were below 1.0. A second and significant elevation in degree of osteogenesis was found at 8 weeks ($P = 0.01$–0.001 compared with both the 4- and 6-week indices). Not all animals examined after 8 weeks, however, showed this increased level of new bone accretion. One group of 20 rats fell within two standard deviations of the 6-week mean value and in this group the mean index of osteogenesis was 1.54. The second group, of eight rats, had higher levels of osteogenesis and gave a mean index of 3.38.

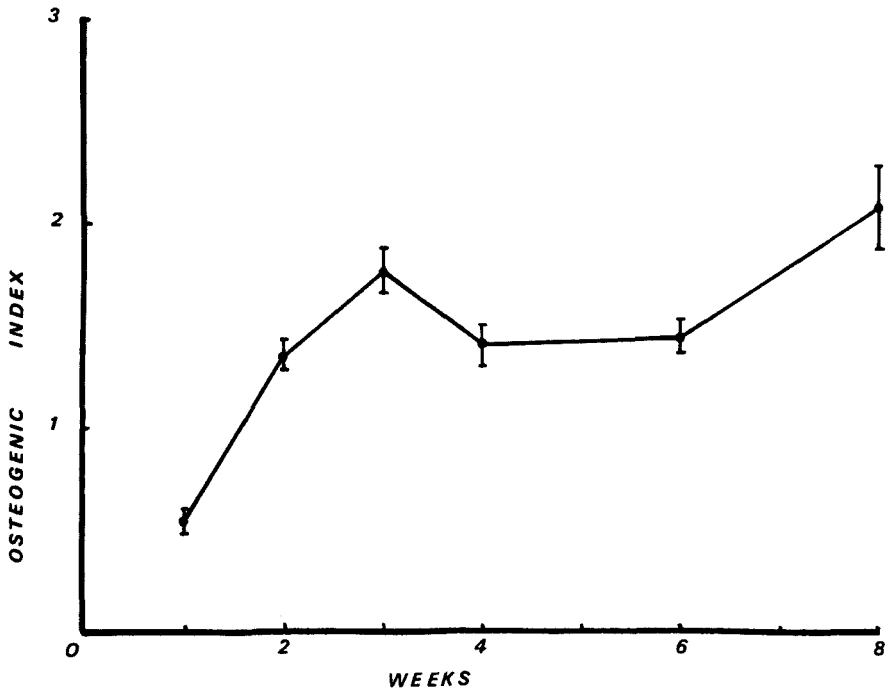

Figure 1. The pattern of osteogenesis in isografts of fresh iliac bone (lines indicate S.E.). Osteogenesis equal to the skeletal level would give an index of 1.

The histological appearances of these cancellous grafts were essentially similar to descriptions given by previous authors (Chalmers 1959, Burwell 1964). They may therefore be summarised briefly. New bone formation was evident by 7 days, by which time the intertrabecular spaces had become filled with granulation tissue and osteoid. By 2 weeks the new bone was well mineralised and had expanded and now occupied many of the original intertrabecular spaces. The original bone of the graft was by this time mostly dead. By 3 weeks the abundant new woven bone was covered by a surface layer of osteoblasts and the spaces in the graft were in many cases showing development of bone marrow. Over the next 5 weeks the graft became progressively smaller and assumed the appearance of an ossicle containing haemopoietic marrow. Over this period, too, the woven bone was gradually replaced by lamellar bone.

Thus there is evidence from the Sr^{85} uptake data of two phases of osteogenesis in bone isografts. The histological method of assessing the grafts could not reveal these two phases. A number of experiments

have therefore been carried out in order to determine the nature of osteogenesis during the early phase in terms of host and graft contributions.

Osteogenesis in preserved bone grafts

Three types of preserved bone grafts have been studied as mentioned above. In the case of both freeze-dried and frozen grafts low levels of strontium uptake were observed at each time interval studied (Figure 2). It was not until 8 weeks after grafting that some grafts in this series exceeded an index of 0.30 which was recommended as the level of significance for uptake by calcium-containing grafts (Elves 1974). The mean index in both frozen and freeze-dried grafts examined after 8 weeks was significantly elevated above the indices at 6 weeks ($P =$ 0.01–0.001 and 0.05–0.01). Histologically, too, these grafts never showed new bone formation during the first 6 weeks, and only small amounts of new bone were present in a number of grafts at 8 weeks. Thus these grafts are completely inert during the early phase, and in the majority of cases also during the later phase.

The freeze-dried calcium-free grafts also showed very low levels of strontium uptake over the first 4 weeks (Figure 2). The mean index in any group did not exceed 0.1 until 6 and 8 weeks after grafting. After 3 weeks only two of 11 grafts had an index above 0.1. Histologically

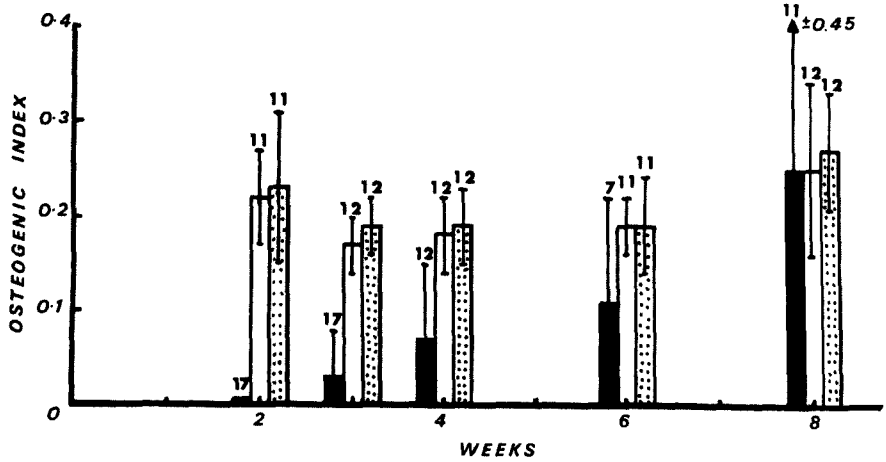

Figure 2. Summary of data showing osteogenesis levels (± S.E.) in isografts of freeze-dried decalcified iliac bone (solid columns), freeze-dried (open column) and frozen bone (stippled column). Number of grafts assessed shown above columns.

new bone was not seen in any graft removed up to 2 weeks after grafting, although many of these grafts showed invasion of matrix by chondrocytes and also small areas of cartilage development. New bone was seen in small amounts in grafts removed after 3 weeks, usually associated with islets of chondrocytes. It was not until 6–8 weeks after grafting that any extensive new bone was seen in and around these grafts. In calcium-free grafts, therefore, the early phase of new bone formation was absent, although there was evidence of second phase osteogenesis.

These experiments suggest a dependence on the graft for the early phase of new bone formation. The graft contribution may be either in the form of osteogenic precursor cells or an "inducer" substance. The preserved grafts used in these studies will certainly lack the former, and it may be argued that an inducer may be destroyed by the treatment.

Osteogenesis in grafts of irradiated bone

Three types of graft were studied for the first phase of osteogenesis (Table 1). Grafts irradiated *in vitro* with 1200 rad showed little osteogenic activity at either 2 or 3 weeks after grafting, and the difference between these grafts and normal control grafts was highly significant

Table 1. Osteogenesis in iliac bone isografts subjected to x-irradiation prior to grafting.

Exp. no.	Treatment of donor	Osteogenic index ± S.E. at* 2 weeks	3 weeks	7 weeks
1	None	1.04±0.094 (9)	2.08±0.20 (5)	1.35±0.256 (6)
	1200 rad *in vitro*	0.21±0.005 (9)	0.43±0.075 (5)	0.74±0.113 (6)
	*P***	0.001	0.001	0.1 —0.05
2	None	2.19±0.170 (5)	1.97±0.08 (6)	—
	850 rad *in vivo*	0.50±0.318 (5)	0.63±0.03 (6)	—
	*P***	0.01—0.001	0.001	
3	None	—	1.32±0.07 (11)	—
	650 rad *in vivo*	—	0.56±0.05 (11)	—
	*P***		0.001	

* Number of rats shown in parenthesis; each rat received one normal and one irradiated graft.

** Student's *t*-test.

at both time intervals. Only two out of nine grafts at 2 weeks, and three out of five examined after 3 weeks gave an index above 0.3. By 7 weeks after grafting a significant osteogenic index was seen in all grafts but in only one instance was the skeletal level of osteogenesis reached. The difference between these grafts and normal control grafts was on the border line of significance. Grafts irradiated *in vivo* with 850 rad all gave a measurable level of osteogenesis 2 and 3 weeks after grafting although none reached an index of 1.0. The grafts were significantly depressed compared with normal fresh grafts. A similar significant depression in degree of osteogenesis was observed in grafts irradiated *in vivo* prior to grafting with 650 rad.

These experiments clearly indicate the dependence of the early phase osteogenesis upon the cellular elements of the graft which must be viable.

Contribution of marrow cells to early phase osteogenesis

The osteoprogenitor cells may reside either amongst the population of cells lining the endosteal surfaces of the graft or amongst the cells of the bone marrow. In order to assess the contribution of the bone marrow, osteogenesis in fresh grafts from which the red marrow had been removed were compared with fresh intact grafts, over the first 3 weeks after transplantation. The results are shown in Table 2. Grafts devoid of bone marrow gave rise to levels of new bone formation which were not significantly different from those in intact grafts after both 2 and 3 weeks.

Table 2. The effect of removal of bone marrow upon the osteogenesis in fresh iliac bone isografts.

Exp. no.	Type of graft	Osteogenic index at* 2 weeks	3 weeks
1	Marrow-containing grafts	1.55±0.118 (5)	2.46±0.233 (6)
	Marrow-free grafts	1.81±0.161 (5)	2.08±0.218 (6)
	P**	0.7	0.3
2	Marrow-containing grafts	1.59±0.120 (6)	1.26±0.111 (6)
	Marrow-free grafts	1.50±0.190 (6)	1.15±0.068 (6)
	P**	0.70	0.40

* Number of rats shown in parenthesis; each rat received one marrow-free and one marrow-containing graft.

** Student's t-test.

DISCUSSION

The bone grafts investigated during this study were placed in a subcutaneous site in order that the pattern of osteogenesis within the graft might not be confused by a fracture healing response on the part of the host. This would have inevitably followed the preparation of an osseous graft bed (Anderson et al. 1965, Puranen 1966). Thus an analysis of the relative contribution of host and graft to osteogenesis is possible, bearing in mind that the host contribution is not likely to be as great as in an osseous site.

By using Sr^{85} for the assessment of osteogenic activity in the fresh cancellous isograft, two phases of osteogenesis are revealed. Histological examination of the grafts can give no indication of these two phases, being a static technique used to study what is essentially a dynamic process. The first phase reaches a peak after about 3 weeks, whilst the second phase occurs somewhat later in approximately one third of the recipients. It should also be stressed that even in those grafts which do not show a marked second peak of osteogenesis the level of osteogenic activity is approximately that in the normal skeleton. Chalmers (1959), in interpreting his histological observations of bone homografts, suggested an early peak in new bone formation during the second and third weeks after grafting, but this was followed by a decline and a second episode of new bone formation occurred 2 months later. Chalmers found that this second phase of osteogenesis was by no means seen in every homograft, and its incidence was of the order of 30 per cent. This figure is very similar to the incidence of late phase increased osteogenesis found in the present study of fresh isografts ($8/28 = 28.6$ per cent), and also to the incidence of osteogenic activity in grafts of dead bone (which is discussed later).

It is clear from the results presented above that the early phase of osteogenesis is dependent upon the viability of the graft. Thus freeze-dried and frozen bone failed to show any early new bone formation. This finding is in agreement with those of others who have used histological methods to examine similar grafts (Ham & Gordon 1952, De Bruyn & Kabisch 1955, Haas 1957, Bonfiglio 1958). Puranen (1966), using the tetracycline marker technique, also found a severe impairment of new bone formation in frozen autografts placed in an intraosseous bed in rabbits, when compared with similar but fresh autografts. Thus in the preserved grafts osteogenesis did not begin until 3 weeks after transplantation and remained feeble. The same author

was able to show that osteogenic potential was lost from autografts if they were exposed to air, and presumably allowed to dry, for an hour. Grafts kept in saline solution also lost their ability to form new bone rapidly after 3 hours of storage; 1 hour of such storage, however, did not affect the graft. The latter two findings were confirmed by Bohr and his colleagues (1968) who placed preserved grafts in either intra-osseous or intra-muscular sites, and also used tetracycline label to reveal new bone. X-irradiation to the bone before grafting also abolished, or reduced, the early phase of osteogenesis as shown above. These findings all point to the requirement for viable cells of graft origin for the initial establishment of osteogenesis.

The second phase of new bone formation is, however, not wholly dependent on the cellular population of the graft as there is evidence of the second phase in grafts of irradiated bone examined after 7 weeks. The host probably provides osteogenic precursor cells which become involved in late phase new bone formation. However, descendents of the original graft cells may also be active in some way in new bone formation in fresh viable grafts during this period, because second phase osteogenesis is not as high in the absence of graft-derived new bone. Puranen (1966) has presented evidence that an autograft of bone, if it is successful in production of new bone, will also stimulate osteogenic activity in the nearby host bone. Inert preserved bone does not have this ability. Thus it may be suggested that the newly formed bone has some osteo-inductive capacity upon the host cells.

The late phase is also evident in decalcified freeze-dried material which showed a tenfold increase in Sr^{85} uptake at 8 weeks compared with those examined after 3 weeks. In the case of the decalcified grafts, strontium uptake was extremely low in most recipients. This is due to the absence of calcium in the graft matrix and therefore the removal of the complication of "deep exchange" which may occur in mineralised bone (Elves 1974). All strontium uptake in these grafts is therefore due to new bone formed in the graft. In four out of 11 recipients of decalcified grafts the osteogenic index was above 0.1 at 8 weeks. Thus, it seems that the second phase of osteogenesis is established in only approximately one third of the recipients by 8 weeks. In the freeze-dried and frozen grafts the evidence for late osteogenesis is less convincing. However, with both materials, grafts are found which show an index of osteogenesis of 0.3 or above after 8 weeks (3/12 in the freeze-dried, 5/12 in the frozen). Thus it may be concluded that the biphasic pattern of new bone formation found by Chalmers (1959) in homo-

grafts also occurs in isografts of cancellous bone but is not revealed by histological techniques.

The origin of the new bone appearing in bone grafts has been a matter of dispute for some years. Thus, on the one hand, there is the school of thought which holds that cells of the autograft bone itself do not survive, and therefore do not contribute to the new bone, which is produced from connective tissue and other cells of the graft bed (Barth 1893, Reynolds & Oliver 1949, De Bruyn & Kabisch 1955). According to this school the bone graft dies and, as it becomes necrotic, liberates substances capable of transforming mesenchymal host cells into osteogenic cells (Levander 1938). On the other hand there are those who hold the view that osteogenic cells in the graft do survive, proliferate and can give rise to new bone formation (Abbot et al. 1947, Hutchinson 1952, Ham & Gordon 1952, Campbell et al. 1953, Puranen 1966, Bohr et al. 1968). Others adopt the view that the new bone arises both by activity of cells in the graft and also by osteoinduction by the graft from host mesenchymal cells (Urist & McLean 1951, Ray & Sabet 1963). The results obtained during the present study indicate that the latter view is most probably correct. First phase osteogenesis clearly has a major graft component, as it is almost completely abolished in dead grafts.

It may be argued, however, that x-irradiation, freezing or drying may destroy the graft's capacity for osteoinduction in the host. In answer to this criticism it is pointed out that in the present study second phase osteogenesis is evident, although poor, in some of these non-viable grafts. Chalmers and his colleagues (1959) have also found histological evidence of new bone formation in freeze-dried and frozen grafts and also in freeze-dried bone exposed to high doses of irradiation. Similarly, Burwell (1966) has reported that frozen, freeze-dried and heavily irradiated homografts of bone all retain their ability to induce new bone formation in autologous bone marrow in composite homograft/autografts.

It may be suggested, therefore, that osteogenesis in autologous or isologous bone grafts is initially derived from cells in the graft which rapidly proliferate and form new bone. Host cells then enter the graft and are induced by the graft to become osteogenic, and these too will ultimately contribute to new bone formation in the graft. Thus the latter part of the first phase is probably of mixed graft and host origin. To what extent the host contribution is dependent upon the initial graft-derived osteogenesis is not clear, although it is found that second

phase osteogenesis is very poor in devitalised grafts. This fact may, on the other hand, indicate that at least part of the late osteogenesis is due to activity of original graft cells or their descendents.

Another enigma surrounds the identification of the cells in the graft which have osteogenic potential. Clinically it is of some importance to solve this problem in order to preserve these cells in bone used for transplantation. Many authors agree that survival of endosteal and periosteal cells after grafting is possible and that these cells are important for new bone formation (Lacroix 1951, Ham & Gordon 1952, Hutchinson 1952, Urist & McLean 1952, Campbell et al. 1953, Bonfiglio 1958, Burwell 1964). Certain cellular components of the bone marrow have also been suggested as osteogenic precursor cells. Bone marrow undoubtedly has osteogenic potential when used as an autograft (Levander 1940, Urist & McLean 1952, De Bruyn & Kabisch 1955, Burwell 1964, Plenk et al. 1972, Salama et al. 1973). From the data presented above it would seem that under the conditions of these experiments the bone marrow is not a major source of osteoprogenitor cells, as its removal has no significant effect on the early phase of new bone formation.

SUMMARY

The pattern of new bone formation has been studied in isografts of fresh iliac bone and also in isografts of dead or irradiated iliac bone. Two phases of osteogenesis have been found in some fresh grafts. An early phase occurs during the first 3 weeks after transplantation, whilst the second phase is found after 8 weeks. The first phase is absent from non-viable grafts and it is therefore concluded that cells of the grafts are largely responsible for this early osteogenesis. The second phase, it is suggested, has a major host component, and may be due to induction of osteogenic potential in host mesenchymal cells. There may be some dependence of the second phase on the first but the extent is not clear. Removal of the bone marrow from the graft has little effect upon the first phase of new bone formation and it is suggested that surviving endosteal cells are the main participants in early osteogenesis.

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Key words: bone grafts; osteogenesis; Strontium^{85}; bone growth; transplantation

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