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OSTEOGENESIS AFTER BONE AND BONE MARROW TRANSPLANTATION

II. The Initial Cellular Events Following Transplantation of Decalcified Allografts of Cancellous Bone

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An experimental study was done in rabbits to investigate the fate of allogeneic iliac cancellous bone, both non-decalcified and decalcified with hydrochloric acid, transplanted to a muscular site for up to 14 days. Some of the treated allografts were impregnated with autologous bone marrow cells, obtained from the femoral medulla by aspiration, and each was compared with allografts alone. Combined myelo-osseous grafts produced bone after 7 to 8 days implantation, as did marrow autografts alone. In addition non-decalcified implants stimulated the production of multinucleated giant cells. Three different types of wash solution were used but these did not influence the cell population seen, nor the new bone formation. It is concluded that the critical events in bone formation after transplantation occur less than 8 days after the transplantation and that marrow cells have osteogenic capacity. This has relevance to the clinical aspects of bone grafting.

Key words: bone; bone transplantation; bone marrow cells; bone cells

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It has previously been shown by Nade & Burwell (1977) that decalcified iliac cancellous bone, impregnated with autologous bone marrow and grafted to a muscular site in rabbits, formed new bone by 14 days after implantation. Also bone marrow autografts *alone* did this. However, decalcified cancellous bone allografts, without bone marrow, produced, on some occasions, very small amounts of new bone but not until 4 weeks after implantation. These findings were at variance with those of Urist's group (Urist et al. 1967) who used cortical bone from the femoral diaphysis in various species and washed the bone in several different ways after decalcification with hydrochloric acid.

In this paper are reported the morphological findings after implanting hydrochloric acid-decalcified allogeneic iliac cancellous bone, washed in three different ways, and impregnated with autologous bone marrow, into the anterior abdominal wall of rabbits for periods of up to 14 days. The critical events leading to osteogenesis take place during this time period.

Table 1. Types of implant used.

Source of bone and treatment	Method of wash	Whether with marrow
Marrow-free, iliac cancellous, non-decalcified	0.1 M NaH_2PO_4	With and without
	70 % ethanol	With and without
	0.9 % NaCl	With and without
Marrow-free, iliac cancellous, decalcified in 0.6 N HCl	0.1 M NaH_2PO_4	With and without
	70 % ethanol	With and without
	0.9 % NaCl	With and without
Nil	Nil	Marrow alone

MATERIAL AND METHODS

The experimental design used was similar to that employed previously for evaluating the effect of impregnating fresh and treated allografts of cancellous bone with autologous red marrow (Burwell 1964, 1966, Nade & Burwell 1977).

The donor bone and methods of treatment

Bone for implantation was taken from both ilia of recently killed rabbits under sterile conditions. The outer cortex was removed, and bone marrow was dislodged from the inter-trabecular spaces using a forceful jet of sterile water. The ilia were then cut into oval shaped pieces, 4 mm long and 2 mm wide, using a stainless steel punch, and the remaining blood and marrow cells were flushed out until the pieces looked quite white. Half of the pieces were then decalcified in a cold room (0° to 4° C) with continual agitation in 5 mm of 0.6 N hydrochloric acid (HCl) for 5 h. The decalcified and the non-decalcified bone were washed twice in one of three solutions: 0.1 M (isotonic) sodium dihydrogen phosphate buffer at pH 7.4; 70 per cent ethyl alcohol; or 0.9 per cent sodium chloride. Wash solutions were decanted and the bone stored, each piece in a numbered, sealed container, by freezing to −20° C.

The preparation and implantation of the treated combined myelo-osseous grafts

The recipients were 42 adult female rabbits. Under anaesthesia, small pouches were opened between the oblique muscles of the anterior abdominal wall, and a single graft was inserted into each pouch. In order to standardize the host response to each type of implant used, 13 implants were inserted into each animal. The types of implant used included iliac bone marrow *alone* and in the form of combined myelo-osseous grafts, the marrow being aspirated from a femur of the recipient immediately before implantation. Table 1 shows the types of implant used. As a control, a single autograft of red marrow was inserted into a separate pouch.

Three animals were killed at daily intervals up to 14 days after implantation.

Retrieval of grafts and preparation for examination

Animals were anaesthetized and killed by intracardiac vascular perfusion with glutaraldehyde through a carotid arteriotomy. The anterior abdominal wall was removed, pinned on a cork board and fixed by immersion in a 2.5 per cent glutaraldehyde solution. After fixation, the abdominal wall was subjected to high definition radiography. Each graft site was then cut out in blocks of about 1 cm square, decalcified and prepared for histological examination, staining 6 μm sections with haematoxylin and eosin. No attempt was made to quantitate the amount of bone which formed, as had been done previously (Nade & Burwell 1977).

RESULTS

Radiological studies

All 252 calcified implants were detected. One hundred and twenty-six decalcified grafts implanted alone did not produce radiological evidence of (re-) calcification.

Decalcified allografts implanted combined with *autologous marrow* showed evidence of calcification from the *eighth* day onwards. This was small in quantity and in the form of scattered specks (Figure 1).

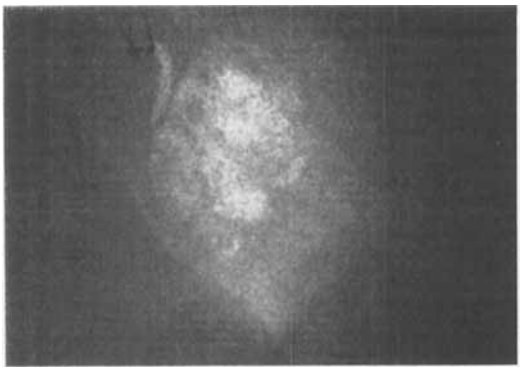

Figure 1. Radiograph of implant site in abdominal wall 12 days after insertion of HCl-decalcified (radiolucent) cancellous iliac allograft combined with autologous marrow. Scattered specks of calcification are seen, which on histological examination (Figure 3) were due to new bone formation (magnification × 6).

Twenty-six out of 42 autografts of marrow alone showed similar specks of calcification, increasing in quantity from the *fourth* day onwards.

Histological studies

All implant sites showed evidence of surgical trauma with haemorrhage and surrounding fibrosis. Haemorrhage was readily visible up to about the seventh day in all implants, and the fibrous capsule and fibroblastic reaction was maximal between 4 and 12 days, in all except the undecalcified grafts inserted alone in which this occurred between 8 and 13 days. No differences were noted in the responses to the three different types of wash procedure.

The morphological features were similar, except for the presence of ovoid cells, to those described in guinea pigs by Cummine & Nade (1977).

The staining of osteocytes within lacunae of the implanted bone, the detection of implanted bone, the presence of osteoblasts and newly formed bone and the presence of giant cells require separate description.

a) *Staining of osteocytes.* There was a difference in the persistence of basophilic nuclear staining of osteocytes in treated allogeneic implanted bone between the decalcified and non-decalcified implants. Such staining could be seen on the day of implantation and the first day afterwards in HCl-decalcified grafts, but was retained until the seventh or eighth day in those not decalcified, but washed before implantation, either combined with marrow or alone.

b) *Detection of implanted bone marrow.* Where implants did not contain bone marrow, no features of marrow cells, or fat spaces were seen. In combined myelo-osseous grafts, and in marrow autografts alone, the marrow portion of the implant was detected by the cellular distribution pattern and interspersed fat spaces (Figure 2). In marrow autografts the implant site was visible up to the fourteenth day—but decreased in intensity from the eighth day. In combined grafts the marrow component was only easily detected until the seventh or eighth day, except in saline washed non-decalcified grafts where it persisted until the twelfth day.

c) *The presence of osteoblasts and newly formed bone.* These two features occurred simultaneously, osteoblasts not

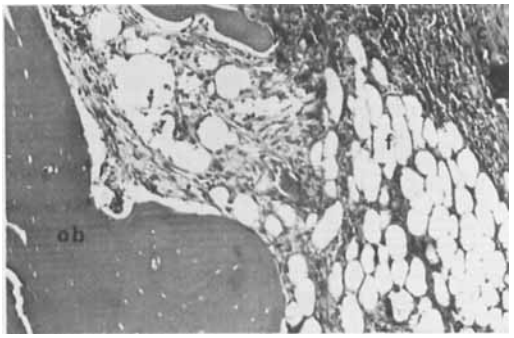

Figure 2. Photomicrograph of undecalcified, saline washed allogeneic iliac cancellous bone implant combined with autologous marrow 6 days after insertion. The marrow component is identified by fat spaces (f) and some cellular aggregates adjacent to the implanted bone (ob) within the fibrous capsule (c). (Haematoxylin and eosin, × 54).

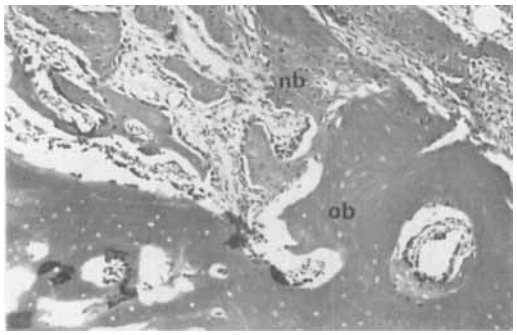

Figure 3. Photomicrograph of HCl-decalcified, phosphate washed allogeneic iliac cancellous bone implant, combined with autologous marrow, 12 days after insertion. The newly formed bone (nb) containing osteocytes and lined by osteoblasts is easily distinguished from the non-viable implant (ob). (Haematoxylin and eosin, × 54).

being recognisable as such until bone had appeared. New bone stained slightly more purple and had osteocytes in lacunae and surrounding matrix (Figure 3). A so-called basophilic cement line formed a line of demarcation in some non-decalcified implants. Without the presence of a marrow autograft, *no* newly formed bone, nor osteoblasts, were seen. In the 42 marrow autogafts inserted alone these features were first seen on the seventh or eighth day after after implantation, and when combined with any allogeneic bone implant, bone did not appear before the eighth day. The pattern was not altered by the method of washing the bone.

d) *Multinucleated giant cells.* These were not seen in marrow autografts, nor around bone which had been decalcified before implantation. In the 252 non-decalcified implants they first appeared about 6 or 7 days after grafting, while in the combined grafts (non-decalcified) they were first noticed after 8 days with ethanol washed implants, 12 days with saline washed and were seen at various times in phosphate washed implants. The significance of these differences is not clear; however, these cells were always seen about extremities of the implant, and usually in Howship's lacunae.

DISCUSSION

The stimulation of osteogenesis and the identification of cells which contribute to the synthesis of bone matrix have been studied for over three centuries. The considerable variation in findings reported has been due to the animal species used, the interpretation of histology, the technical differences in laboratories and the acceptance of conclusions from statistically insignificant figures.

In the interpretation of previously described work, one must be careful if one accepts the criterion of nuclear staining of osteocytes as indicating cell viability, for the findings of this study as well as of Yoshiki (1962) and Burwell (personal communication 1970) show that such a stain in non-decalcified stored bone may be retained for up to 7 days, when the cells are by all accepted criteria non-viable.

Previous studies (Nade 1970, Nade & Burwell 1977) have failed to confirm the findings of Urist's group (Urist et al. 1968) that HCl decalcified allografts of bone implanted into an intermuscular site evoke the production of newly formed bone, presumably by the process of induction. One variable was the method by which the bone was washed after decalcification. This study shows that the cellular response, in the rabbit, is not influenced by the use of sodium chloride solution, 70 per cent ethanol or an isotonic phosphate buffer. Admittedly, Urist's group have used diaphyseal cortical bone, while this study has used iliac cancellous bone; however, Cummine & Nade (1977) showed that the cellular response around these two types of bone is not different (in the guinea pig).

The findings of earlier reports have been confirmed, and have led to the conclusion that fresh bone marrow auto-

grafts are osteogenic (Bruns 1881, Danis 1956, Amsel & Dell 1971) and that marrow cells produce bone in the presence of decalcified allografts of cancellous bone (Burwell 1966, Pike & Boyne 1974). Whether such bone growth is stimulated by the allograft (Sabet et al. 1964, Boyne 1970, Buring 1975) or whether it acts purely as a scaffold to retain the marrow autograft *in situ* (Burwell 1966) has not been determined. There is a critical number of cells per unit volume (packing density) below which osteogenesis does not take place (Friedenstein et al. 1966, Amsel & Dell 1971). However, the newly formed bone, and adjacent osteoblasts were present on the eighth day after implantation in this study, so that the critical events in the stimulation of osteogenesis occur well before that time has elapsed.

Multinucleated giant cells were yet again (Nade 1970, Nade & Burwell 1977, Cummine & Nade 1977) seen around non-decalcified grafts, suggesting that the cellular morphology around skeletally derived implants is influenced by their physical and/or chemical nature (Young 1966). The significance of such giant cells is unknown; presumably they have a role in demineralization of hard pieces of bone, and by doing so, deplete the cell population surrounding an implant of potentially osteogenic cells.

Although it has been suggested that the influence of skeletally derived tissues is at least partially masked or inhibited when bone is mineralized (Morris 1973, Nade & Burwell 1977) the time at which bone formed was not altered by the failure to decalcify although no attempt was made to assess the quantity of bone that formed.

It has previously been shown (Bloom et al. 1941, Tavassoli & Crosby 1968, Nade 1970, 1973) that cells within the bone marrow population are osteogenic. Whether transplanted cells contribute directly by division and differentiation or whether they degenerate and their breakdown products stimulate bone formation in the surrounding connective tissue has not been clarified and advocates of the former (Dorr et al. 1962, Friedenstein et al. 1968, Danis 1973) and latter theories (Crosby 1970, Buring 1975) exist. Certainly by light microscopy, one is unable to identify in the first 7 days after implantation those cells which are about to synthesize bone matrix. Cummine & Nade (1977) have described ovoid cells in guinea pigs which appear adjacent to bone implants about the time that osteogenesis commences, but similar cells have not been detected in the rabbit. Perhaps these are the primitive mesenchymal cells, or reticular cells described by Amsel & Dell (1971). Electron microscopy would be a useful tool in determining which cells in the first 7 days after bone marrow transplantation appear to be assuming osteoblast characteristics and bone matrix synthetic activity.

Similar findings have now been described in rabbits of a different strain to those in this study (Nade & Burwell 1977), in the rat (Burwell 1966), the monkey (Boyne 1974), the dog (Newman & Boyne 1971), the guinea pig (Cummine & Nade 1977) and man (Marble et al. 1970, Boyne 1974) under various experimental conditions.

The formation of bone by marrow as a fundamental response to injury or insult of that tissue has been proposed (Cummine & Nade 1975) and the ability to control this response by the surgeon should make it possible to stimulate osteogenesis when and where it is required without the need for taking massive bone autografts (Nade 1970, Boyne 1973).

Decalcified allogeneic bone, preferably cancellous for its cell-retaining mesh of trabeculae, could be stored easily in sterile containers by freezing or lyophilisation and used as a relatively non-anti-

genic vehicle for implantation of osteogenic bone marrow—and possibly act as a stimulus to its osteogenic capacity. Despite previous attempts to provide 'bone-banks' this hypothesis changes emphasis from the cellular to an environmental role for the skeletally derived graft.

Studies of the re-construction of mandibular defects using such combined myelo-osseous grafts isolated from surrounding cells by a porous membrane (Boyne 1969, Richter & Boyne 1969) have suggested the direct involvement of such marrow cells. Mitchell & Marble (1974) do not believe that the calcified matrix of cancellous bone produces any significant osteogenic effect, but acts as a restraining lattice for the marrow cells.

With the realization that living autografted bone marrow cells are osteogenic, clinical trials should be undertaken to assess the potential of this type of 'boneless bone grafting'.

SUMMARY

1. This paper reports a radiological and histological study of the fate of hydrochloric acid-decalcified allogeneic iliac bone impregnated with autologous bone marrow and grafted to a muscular site in rabbits for up to 14 days.

2. After decalcification, the bone pieces were washed in either 0.9 per cent sodium chloride solution, 70 per cent ethanol, or 0.1 M isotonic phosphate buffer at pH 7.4. The treated pieces of bone were sealed in individual containers and stored by freezing. Non-decalcified bone, similarly washed, was used as a control in the same animal.

3. Bone marrow was obtained by aspiration from the femoral medullary cavity through a drill hole just before implantation and combined with the treated and stored allogeneic bone.

4. Autografts of bone marrow alone produced bone after 7 to 8 days implantation, as did combined myelo-osseous grafts.

5. In the absence of bone marrow no newly formed bone was seen.

6. Non-decalcified implants stimulated the production of multi-nucleated giant cells about them.

7. The type of wash used did not influence the cell pattern seen around the grafts, nor the new bone formation.

8. The retention of nuclear staining by osteocytes cannot be accepted as evidence of cell viability.

9. The results of these and similar experiments using combined grafts of bone and marrow show that bone marrow autografts are osteogenic and this capacity may be controlled by the surgeon by varying the microenvironment in which the marrow cells are placed.

10. The critical events in bone formation after marrow injury by transplantation occur less than 8 days after such transplantation.

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