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OSTEOGENESIS AFTER BONE AND BONE-MARROW TRANSPLANTATION

I. Studies with Combined Myelo-osseous Grafts in the Guinea Pig

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There has been conflicting evidence in the past regarding the contribution made to new bone formation by various components of a bone graft. This study in guinea pigs has compared cortical and cancellous bone allografts, both non-decalcified and hydrochloric acid decalcified, inserted into a muscular site for up to thirteen days, both alone and combined with autologous bone marrow. New bone formation was seen *only* if the implant contained fresh autologous marrow and it was not seen before the ninth day of implantation. There were no differences in the cell populations about cortical and cancellous grafts. Undecalcified bone evoked giant cell formation as well as new bone.

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

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The stimulation of osteogenesis by combined myelo-osseous grafts has been used clinically (Boyne 1973) and experimentally (Pike & Boyne 1974) in several species.

Results from bone allografts alone have been conflicting (Nade & Burwell 1976) and the contribution of the type of bone (cortical or cancellous) or its nature (calcified or decalcified) to osteogenesis after transplantation with marrow needs further investigation.

Studies on osteogenesis in the guinea pig, using bladder mucosa (Huggins 1931) and other transplants (Bang 1973) have been reported, but the combined myelo-osseous graft (Burwell 1964) has not previously been used in that animal.

The morphological changes after bone and bone-marrow grafting in guinea pigs are described in this paper. During the first two weeks after transplantation, new bone formation is dependent on autologous marrow. Whether the implant is calcified or decalcified does not influence osteogenesis, but giant cells are again seen around calcified implants (Nade 1970, 1973, 1976). These results are similar to earlier findings in the rabbit and rat.

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Table 1. Types of implant used.

Source of marrow-free allogeneic bone	Treatment before freezing for storage	Whether with marrow
Iliac cancellous	Non-decalcified	Alone
	Non-decalcified	Combined
	HCl-decalcified	Alone
	HCl-decalcified	Combined
Femoral diaphyseal cortical	Non-decalcified	Alone
	Non-decalcified	Combined
	HCl-decalcified	Alone
	HCl-decalcified	Combined
Nil	Nil	Autologous marrow Alone (2 grafts)
	Total	10 implants

MATERIALS AND METHODS

The experimental design was similar to that used by Salama et al. (1973) and Nade & Burwell (1976), except that: (i) the species studied was the guinea pig; (ii) the time period studied was up to two weeks; and (iii) autologous marrow was obtained from the femoral medullary cavity by needle aspiration through the opened knee joint.

(a) *Donor bone*

Cortical bone was obtained from the diaphyseal femora and cancellous bone from the ilia of killed guinea pigs. The bone for implantation was cut into pieces about 5 millimetres square. Both types of bone were decalcified in 0.6 N hydrochloric acid at 2° C with continuous agitation, the cortical bone for 4 hours, and the cancellous for 1.5 hours.

(b) *Insertion of implants*

The recipients were male guinea pigs bred by the University of Sydney. Under anaesthesia the fibres of the external oblique muscle of the anterior abdominal wall were exposed, a pouch opened between these fibres, and the graft inserted. The pouch was closed with a silk stitch. Ten implants were inserted into each animal—one of each of the allografts alone, and two pieces of bone-marrow autografts served as controls for the combined myelo-osseous grafts. Table 1 shows the types of implants used.

Retrieval of implants was made at 1, 3, 5, 7, 9, 11 and 13 days after insertion, by killing the animal. Three animals were used for each time period.

RESULTS

Table 2 shows the number of implants for each time period, the nature of the implants, the retrieval rate for similar implants at each time period, and new bone formation rates.

The histology is discussed in three groups:

(a) Bone implants alone;
(b) Bone implants combined with autologous marrow;
(c) Marrow alone.

(a) *Bone alone*

There was no major difference in the response seen after cancellous or cortical allogeneic bone implants.

Day 1: The osteocyte lacunae were empty. Extravasated red cells and fibrin were seen about the implants.

Day 3: Some early cellular invasion of the implant was seen, more conspicuous on the trabeculae of cancellous implants. A mild inflammatory response was noted in some sections.

Table 2.

Implant	Number implanted	Number retrieved	Number with new bone	Number implanted	Number retrieved	Number with new bone	Number implanted	Number retrieved	Number with new bone
Duration (days)	Control All allogeneic grafts alone (Not combined with marrow)			Non-decalcified cortical implants combined with autologous marrow			HCl-decalcified cortical implants combined with autologous marrow		
1	12	10	–	3	3	–	3	3	–
3	12	10	–	3	2	–	3	3	–
5	12	8	–	3	1	–	3	1	–
7	12	7	–	3	3	–	3	2	–
9	12	7	–	3	2	2	3	3	3
11	12	8	–	3	1	1	3	3	1
13	12	10	–	3	3	3	3	2	1
	Control Bone marrow autografts alone			Non-decalcified cancellous implants combined with autologous marrow			HCl-decalcified cancellous implants combined with autologous marrow		
1	6	4	–	3	2	–	3	2	–
3	6	3	–	3	3	–	3	2	–
5	6	4	–	3	1	–	3	1	–
7	6	3	–	3	2	–	3	1	–
9	6	2	2	3	3	2	3	3	1
11	6	3	2	3	1	1	3	2	1
13	6	3	2	3	3	2	3	3	2

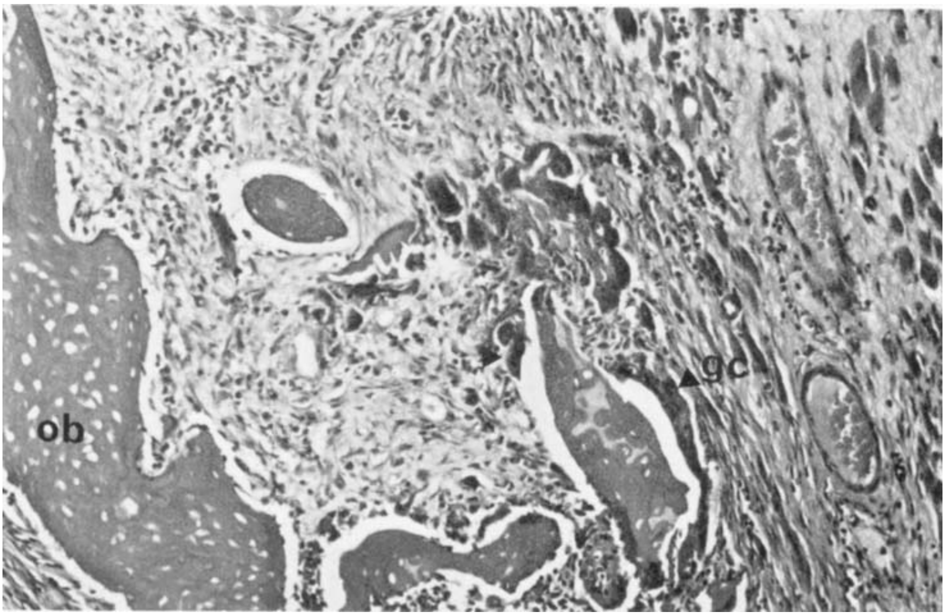

Figure 1. The osteocyte lacunae of the implanted undecalcified allogeneic cancellous graft (ob) are empty. In the centre of the field are several multinucleated giant cells (gc) arranged about a bone spicule. Fibrous tissue cells and stroma fill the intertrabecular interstices. (Implant duration 9 days, haematoxylin and eosin, × 100).

Day 5: Most implants showed evidence of connective tissue cell invasion. The degree of fibroblast infiltration appeared greater with cancellous implants.

At the periphery, the fibroblasts were arranged circumferentially. Giant cells were seen around cortical and cancellous decalcified implants. Apparent now in smaller numbers than in combined grafts were large ovoid mononuclear cells showing a round or oval, pale-staining nucleus with variable but usually faint basophilia, some had a nucleolus, a large nuclear/cytoplasmic ratio, and scanty, poorly eosinophilic cytoplasm whose boundary was often indefinable. These cells appeared more plentiful in the graft interstices, but could be seen mingled with the fibroblasts. Occasional polymorphs were noted.

Day 7: The pattern established at day 5 persisted, but showed increased fibroblast and collagen formation with encapsulation of the implant, and increased numbers of giant cells around non-decalcified implants (Figure 1).

Days 9, 11, 13: No significant changes occurred. The final picture was that of a fibrous tissue encapsulated implant. The ovoid cells seen at day 5 became progressively less in number.

In summary, for all bone allografts implanted alone:

- No new bone was seen,
- Giant cells formed only around non-decalcified implants,
- No cartilage cells were seen.

(b) *Combined allogeneic bone and autologous marrow*

There were no differences noted between cortical and cancellous bone.

Days 1 and 3: Early fibrous invasion was evident in the interstices of cancellous bone. The number of red cells was variable and probably related to the trauma of implantation. Fibrin was

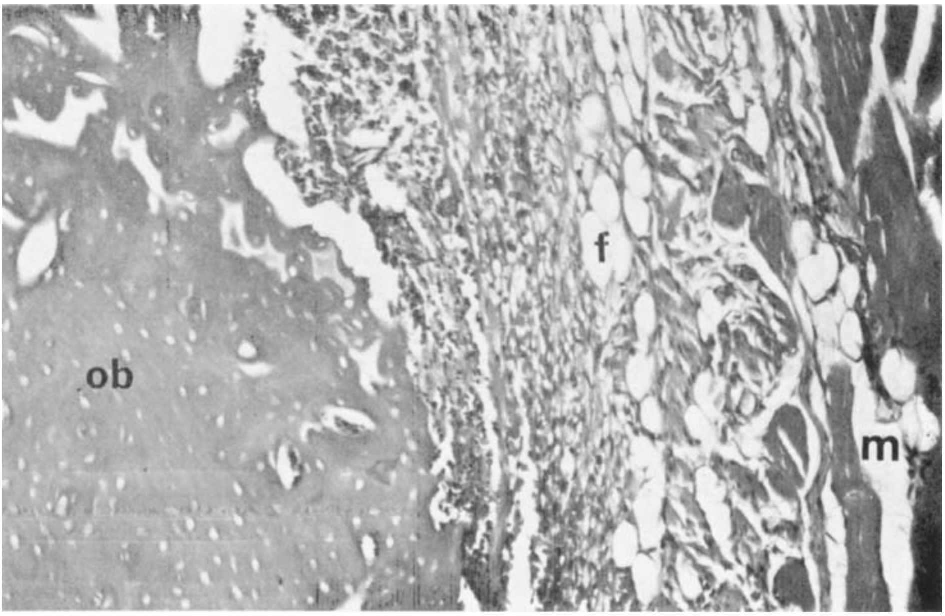

Figure 2. The osteocyte lacunae of the implanted HCl-decalcified allogeneic cortical bone (ob) are empty. Adjacent to this bone is the evidence of combined autologous marrow seen as empty fat spaces (f) and cells forming a mild inflammatory reaction. Surrounding muscle (m) is seen outside the implanted area. (Implant duration 5 days, haematoxylin and eosin, × 100).

usually noted. Signs of a mild inflammatory response could again be detected at the junction of implant and traumatised muscle. Marrow cells were sometimes seen, but as above, the presence of fat spaces was the prime guide to the transplanted marrow.

Day 5: Fibrous tissue infiltration increased, encapsulating the transplant. Fat spaces were seen in some implants (Figure 2). Giant cells were present only in association with non-decalcified grafts.

Compared with bone alone there was an abundance of the large ovoid cells described above. They were seen throughout the cancellous interstices especially, but also in the fibrous capsule mingled with the fibroblasts.

Day 7: Apart from the impression of circumferential orientation of fibroblasts and collagen, and the abundance of ovoid cells, no difference compared with the fifth day was noted. Implanted marrow was not identifiable.

Day 9: New bone was seen in two thirds of the implants. It formed in one or two foci, exhibiting a trabecular pattern in the majority. Usually one part of the new bone abutted the implant on the cancellous trabeculae with cancellous implants, and on the concave surface of the cortical implant (Figure 3). In parts, palissading osteoblasts were seen. The new bone was always easily identifiable when present. It was more basophilic, showed a "cement line" and its lacunae were small and filled by viable osteocytes.

The ovoid cells were very prominent, particularly in association with the new bone. Giant cells were only seen around non-decalcified implants and never related to the newly forming bone.

Days 11 and 13: Progressive fibrous and cellular infiltration of implant interstices and fibrous encapsulation was seen. The new bone was clearly recog-

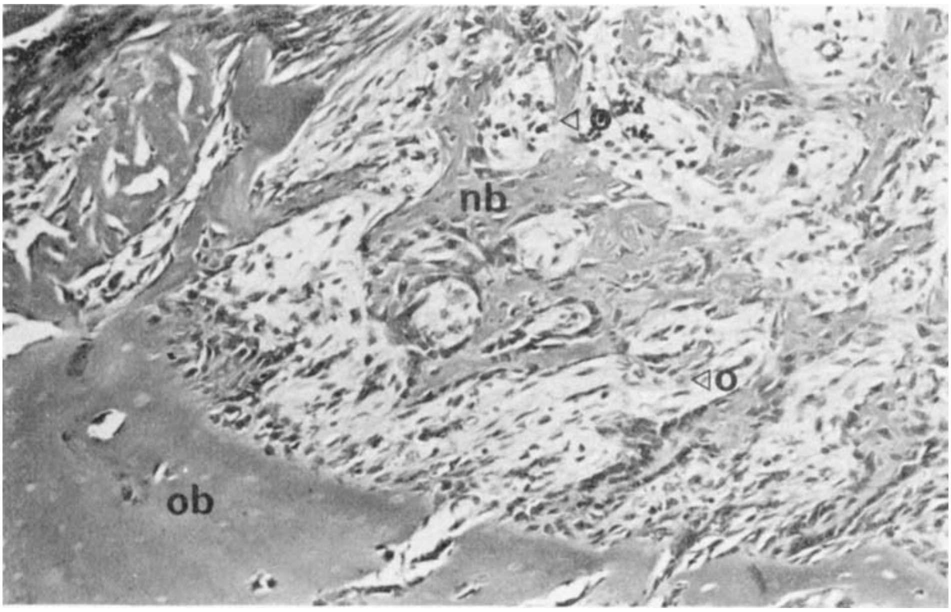

Figure 3. Newly formed bone (nb) abuts the implanted HCl-decalcified allogeneic cortical graft (ob) which was combined with autologous marrow at the time of implantation. The new bone stains more deeply, contains osteocytes and is lined by osteoblasts. Ovoid cells (o) are seen amid the osteoblasts, fibroblasts and collagenous fibres. (Implant duration 9 days, haematoxylin and eosin, × 100).

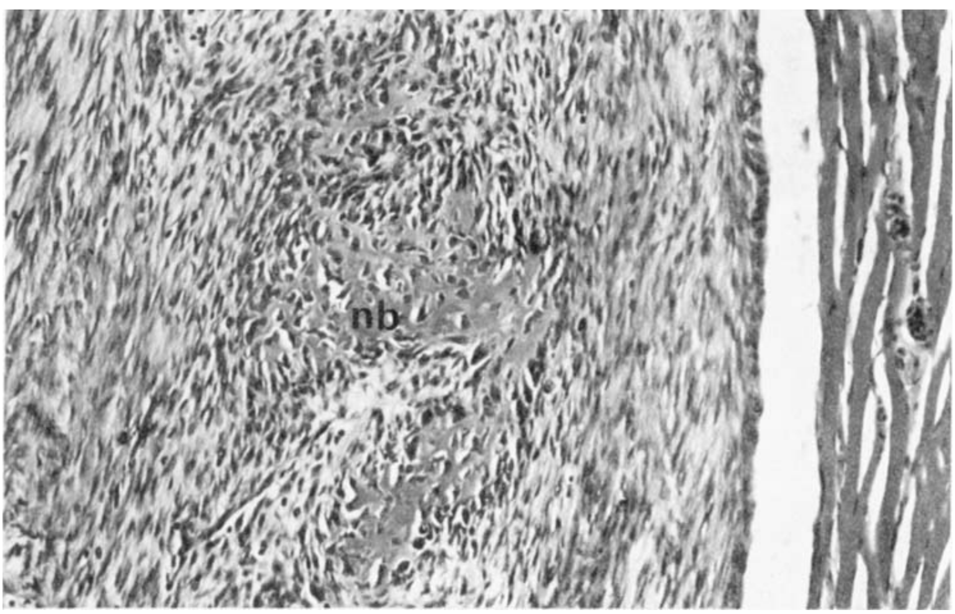

Figure 4. Newly formed bone (nb) and osteoblasts are seen at the site of an autologous bone-marrow graft 9 days after implantation. It is surrounded by fibrous connective tissue and bone-marrow cells are not identifiable. The muscle bed is adjacent. (Haematoxylin and eosin, × 100).

nised. Near the trabeculae of new bone ovoid cells were plentiful.

Among the newly formed trabeculae, numerous blood vessels were seen. Giant cells were again only seen around non-decalcified implants; they were never associated with the new bone.

Where the new bone did not form, the appearances were the same as described under bone implants alone.

(c) *Bone-marrow autografts alone*

At those sites where bone formed the sequence of cellular events followed the same pattern as that described under combined grafts except –

(i) No bone implant was present;
(ii) No giant cells were seen;
(iii) The number of implants forming new bone was less than in the combined grafts (see Table 2);
(iv) The formation of a fibrous capsule was less evident (Figure 4).

In the implants not forming new bone, when the implant site was apparently located, a mixed fibrous and cellular nodule was seen.

DISCUSSION

The history of bone-graft surgery is long and the publications on this subject are voluminous. Although many attempts have been made to produce a material that would serve a useful function in a "bone bank", none has stood the test of time. Indeed, advances in surgical and anaesthetic techniques, and modern metallurgy have given us the means to provide rigidity in places where bone is deficient, and the majority of surgeons appear to favour the use of fresh autografts of cancellous bone for osteogenic function, since their introduction by Matti (1931), Mowlem (1941) and Abbott et al. (1942).

Such grafts are in fact myelo-osseous combinations and the current series of investigations is an attempt to isolate the functionally osteogenic part of the graft. In this way further approaches to a "bone bank" may be possible—perhaps with the emphasis changed from the skeletal derivation of the material, which inevitably dies, to the stimuli which provoke active osteogenesis by living cells.

The osteogenic potential of bone marrow

The fomation of bone in sites of autotransplantation of bone marrow is well established (Danis 1956, 1959, 1960, 1973, Chalmers 1959, Burwell & Gowland 1961, Tavassoli & Crosby 1968, Nade 1970, Maniatis et al. 1971, Patt & Maloney 1972). Although the tissue into which the marrow is transplanted has an influence on the formation of bone after such transplants, Danis (1956, 1960) was able to find newly formed bone on all sites that he studied. The results reported in this paper show that marrow autografts to a muscular bed produced bone in 6 out of 42 grafts (14 per cent). If the graft duration was greater than 7 days, bone was found in 6 out of 18 grafts (33 per cent). Yields of this order have also been recorded by Burwell (1964) in rats, and Nade (1973) has shown a higher yield in rabbits. The reasons for variability in the yields of new bone after marrow autografting include:

(a) difficulty in identification of the site of marrow implantation despite serial sectioning; only 8 out of 18 implants were retrieved, of which 6 (75 per cent) formed new bone, similar to the studies of Danis;
(b) the possibility that cells may have disseminated from the implant site, either by active migration or as a result of muscle contraction. It may be that a minimal packing density of cells is required before bone forms (Friedenstein et al. 1966, Amsel &

Dell 1971 a). Bone implants may assist by holding sufficient marrow cells in the implanted site to allow bone formation (Burwell 1964);

(c) There may be a phase between marrow implantation and repopulation as such during which the response to surgical trauma is no longer apparent (Maniatis et al. 1971);

(d) The possible effect of inductors or repressors of osteogenesis in the micro-environment of the transplanted marrow (Urist 1965, Nade & Burwell 1976). This study confirms that autografts of living bone marrow are osteogenic when placed into a muscular bed. Combination of autologous marrow with chemically treated, dead allografts of bone significantly increased the number of sites in which bone formed.

Does the type of allogeneic bone or its treatment influence osteogenesis?

Because of their inability to reproduce the results of Urist's group, Nade & Burwell (1976) suggested that there may be differences in the influence that cortical and cancellous allogeneic bone have on bone-marrow osteogenesis. Decalcified bone as a stimulus for osteogenesis was first suggested and used by Senn (1889) and subsequent studies have shown that decalcification with hydrochloric acid may be the most effective way of producing an implant which stimulates osteogenesis (Burwell 1966, Urist et al. 1968).

In this series of experiments in the guinea pig, there has been *no* (qualitative) difference shown between cortical and cancellous bone as the allogeneic component of the myelo-osseous grafts, *and also* there was no difference, in terms of new bone formation, between implants which had been decalcified with 0.6N HCl or left undecalcified.

The time of the first appearance of new bone

New bone was not seen in animals killed on the seventh day after implantation, but on the *ninth* day it had appeared. Therefore, for such an appearance of woven bone, recognisable as a tissue, the critical programming event in cell specialisation, be it differentiation of a previously uncommitted cell type, or transformation by induction, or redifferentiation, must occur in the first few days after marrow transplantation. This time sequence is not different from that found in the rabbit (Nade 1976), but contrasts with the findings of Urist et al. (1972) who stated that the guinea pig was a slower bone former than other species. However, his group has experimented with bone allografts alone and claimed that an inductive process due to a component resident within decalcified bone evoked bone formation in a muscle bed 24–28 days after implantation in the rabbit. New bone formed after marrow autotransplantation forms much earlier and the cellular interactions involved may be quite different.

The cells seen about the implants

(a) Multinucleated giant cells were seen only about non-decalcified implants —either cortical or cancellous. They were apparent as early as the fifth day and were located only about the implant and never in relation to newly formed bone. The early appearance of these cells in a non-sensitised animal makes a specific immune rejection mechanism unlikely. Why these cells are seen remains an enigma, but could relate to the chemical or physical composition of the allograft.

(b) The cells described as ovoid cells appearing from the fifth day onwards may be the same as those called primitive mesenchymal cells, reticular cells, or pre-osteoblasts by previous workers (see Amsel & Dell 1971 b). They appeared more plentiful in combined myelo-osseous grafts, and in those implants forming bone were abutting the newly formed bone. Radioisotope studies or electron microscopy (Thorogood & Gray 1975) could help in their identification and warrants further study.

SUMMARY

1. Histological study was made of the fate of bone allografts, impregnated with autologous marrow and grafted to a muscular site in guinea pigs for 1 to 13 days.
2. Four principal types of marrow-free allogeneic bone were evaluated:

   (a) Iliac, cancellous bone, non-decalcified,
   (b) Iliac, cancellous bone, decalcified with HCl,
   (c) Femoral cortical bone, non-decalcified,
   (d) Femoral cortical bone, decalcified with HCl.

   Each of the four types was frozen for storage and later implanted with and without autologous marrow which was obtained from the recipient's femoral medullary cavity.
3. New bone formation was only seen if the implant contained autologous marrow. The new bone was not seen before 9 days of implantation.
4. Autografts of marrow *alone* formed bone in 6 out of 8 sites after 9 days.
5. There was no difference in the cell populations that formed about cortical or cancellous allografts.
6. Undecalcified bone, cortical or cancellous, evoked giant cell formation, as well as new bone.

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