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Fate of Mineralized and Demineralized Osseous Implants in Cranial Defects

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Summary. We have evaluated the fate of mineralized and demineralized osseous implants placed into cranial defects in rats. By 2 weeks, 100% of the defects that had been filled with demineralized bone powder (DBP, 75-250 μm) showed bony repair as judged by histomorphometric analysis and incorporation of ^{45}Ca. The DBP was not appreciably resorbed but rather was amalgamated within the new bone. Histomorphometric evaluation of osteogenesis induced by equal masses of demineralized bone powders of various particle sizes (< 75, 75-250, 250-450, > 450 μm) revealed that the smaller particles induced more bone per field than did the larger particles.

In contrast, mineralized bone powder (BP) was completely resorbed by 3 weeks, without bony repair of the cranial defect. These specimens contained large multinucleated cells and connective tissue.

Implants of bone minerals were also evaluated. Bone ash and deorganified bone powder were surrounded by multinucleated cells within 7 days and completely resorbed by 3 weeks.

It is concluded that (a) demineralized bone powder predictably induces osteogenic healing of cranial defects, (b) demineralized bone powder is not appreciably resorbed prior to bone induction, (c) the extent of bone induction is a function of the surface area of the demineralized bone implant, and (d) mineralized bone powder undergoes obligatory resorption.

Key words: Bone matrix — Induced osteogenesis — Mineral — Bone resorption.

In 1931 Huggins [1] reported that proliferating mucosa of kidney, ureter, or bladder induced bone formation in connective tissue. This was the first experimental model of induced ectopic osteogenesis. More recently, Urist [2] and Reddi and Huggins [3] demonstrated that osteogenesis could also be induced by the devitalized, demineralized matrix of bone or dentin. It has been shown that physical factors, including surface charge and geometry of the matrix, are involved [4]. There is some evidence that a soluble factor from demineralized bone is osteoinductive [5]. In 1889 Senn [6] showed healing of experimental canine calvarial defects and of human tibial and femoral defects with decalcified ovine bone. Others have shown bone formation in periapical areas in dogs and monkeys [7] and in skull defects in rats [8] after implantation of demineralized bone; we have recently demonstrated the usefulness of demineralized implants for bone repair and construction in the craniofacial region in rats [9].

In this study, we quantitated osteogenesis induced by demineralized bone powder and evaluated the fate of demineralized and mineralized implants of comparable particle size and similar processing. Host cellular responses to the two types of implants were also examined.

Materials and Methods

Defects. After 28-day-old rats (male, CD strain, Charles River Breeding Laboratories) were anesthetized with ether, the pericrania were stripped off the parietal skulls through coronal incisions. A 4 mm diameter defect was made through each parietal bone with an electric drill and burr. The defects were rinsed with Ringer's solution and were filled with different implants or left empty. The skin incisions were closed with interrupted sutures. Each group consisted of 25 rats with two defects each.

Implants. Isogeneic bone powder (BP) was prepared from femora and humeri of adult rats. The cleaned diaphyses were extracted with absolute ethanol followed by anhydrous ethyl ether. The bones were pulverized in a Spex liquid nitrogen impacting mill and sieved to particle sizes < 75, 75-250, 250-450, and > 450 μm.

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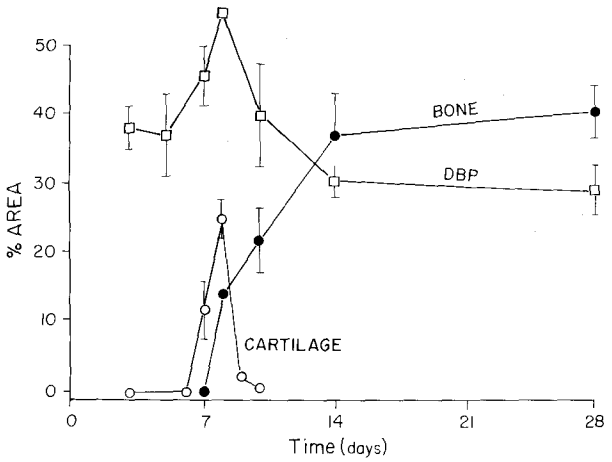

Fig. 1. Histomorphometric analysis of percent area of demineralized bone powder (DBP), cartilage, and bone in cranial defects

Demineralized bone powder (DBP) was prepared by extracting BP with 0.5 *M* HCl (25 meq/g bone) for 3 h at room temperature followed by washes in distilled water to remove all acid and calcium and sequential 60 min washes in absolute ethanol and anhydrous ether.

Ash was prepared by heating pulverized dehydrated bone powder overnight at 600°C.

Deorganified bone was prepared by cold treatment of dehydrated bone powder with 5% NaOCl.

Hydroxyapatite was purchased from Bio-Rad Laboratories, Richmond, California.

^{45}Ca *Incorporation.*

De novo calcification of the cranial defects was quantitated by ^{45}Ca uptake studies in 48 rats. 100 μCi $^{45}CaCl_2$ per 0.1 ml was administered intraperitoneally 4 h prior to sacrifice of the animals. The implantation sites were excised, weighed, and extracted for 30 min in 10 ml of 0.1 *M* $CaCl_2$ at room temperature. The samples were then extracted in 10 ml 0.5 *M* HCl for 3 h. The amount of ^{45}Ca which had been incorporated into the acid-soluble fraction (hydroxyapatite) of the implanted defect was measured by scintillation counting and expressed as cpm/mg tissue.

Histologic evaluation of undemineralized specimens by (a) von Kossa staining for mineral and (b) fluorescence microscopy of sections from animals that had been treated with 15 mg/kg oxytetracycline [9] revealed that only the induced living bone was mineralized. Inasmuch as the particles of implanted demineralized bone failed to become mineralized, the incorporation of ^{45}Ca is a measure of osteogenesis, a conclusion in agreement with the reports of others [3, 10, 11].

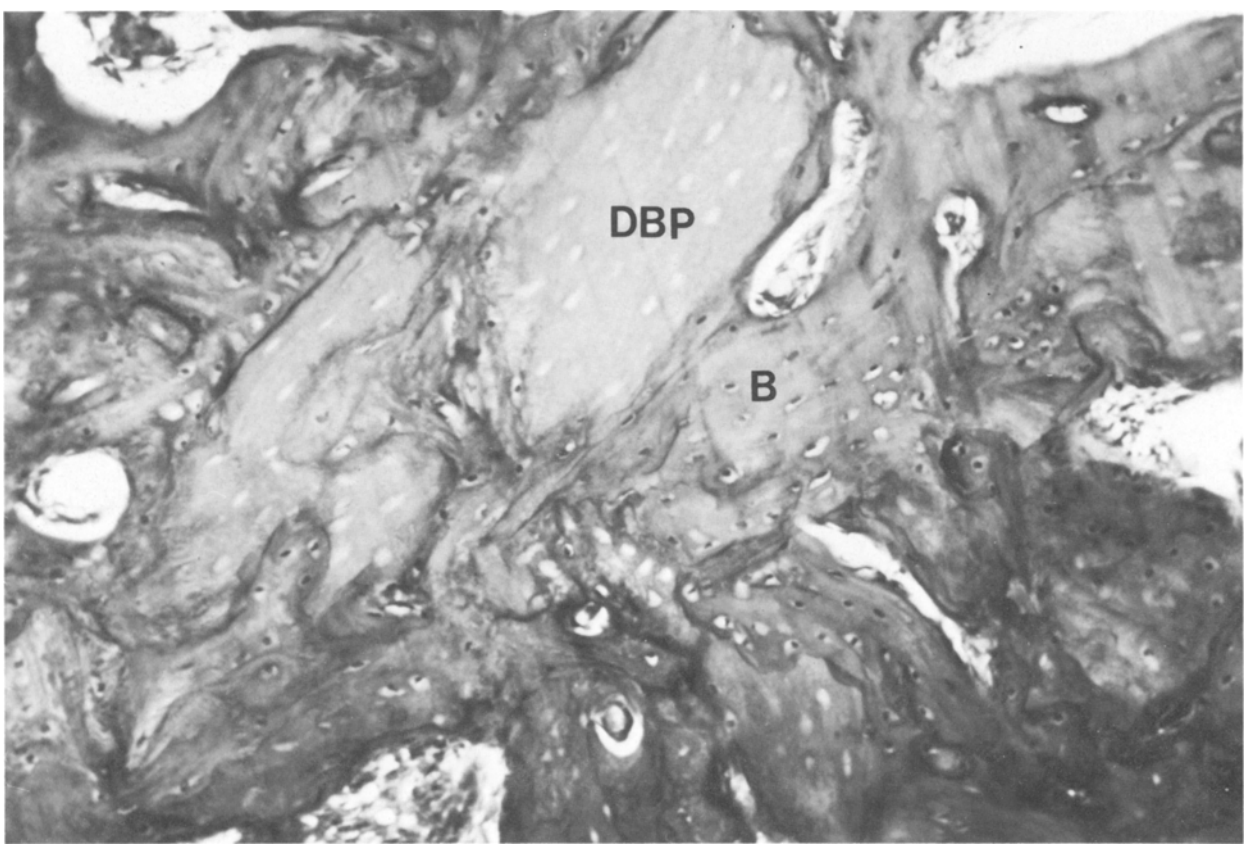

Fig. 2. Induced bone (*B*) with amalgamation of demineralized bone powder (*DBP*) 6 months after implantation into cranial defect. Safranin-O. 640× magnification

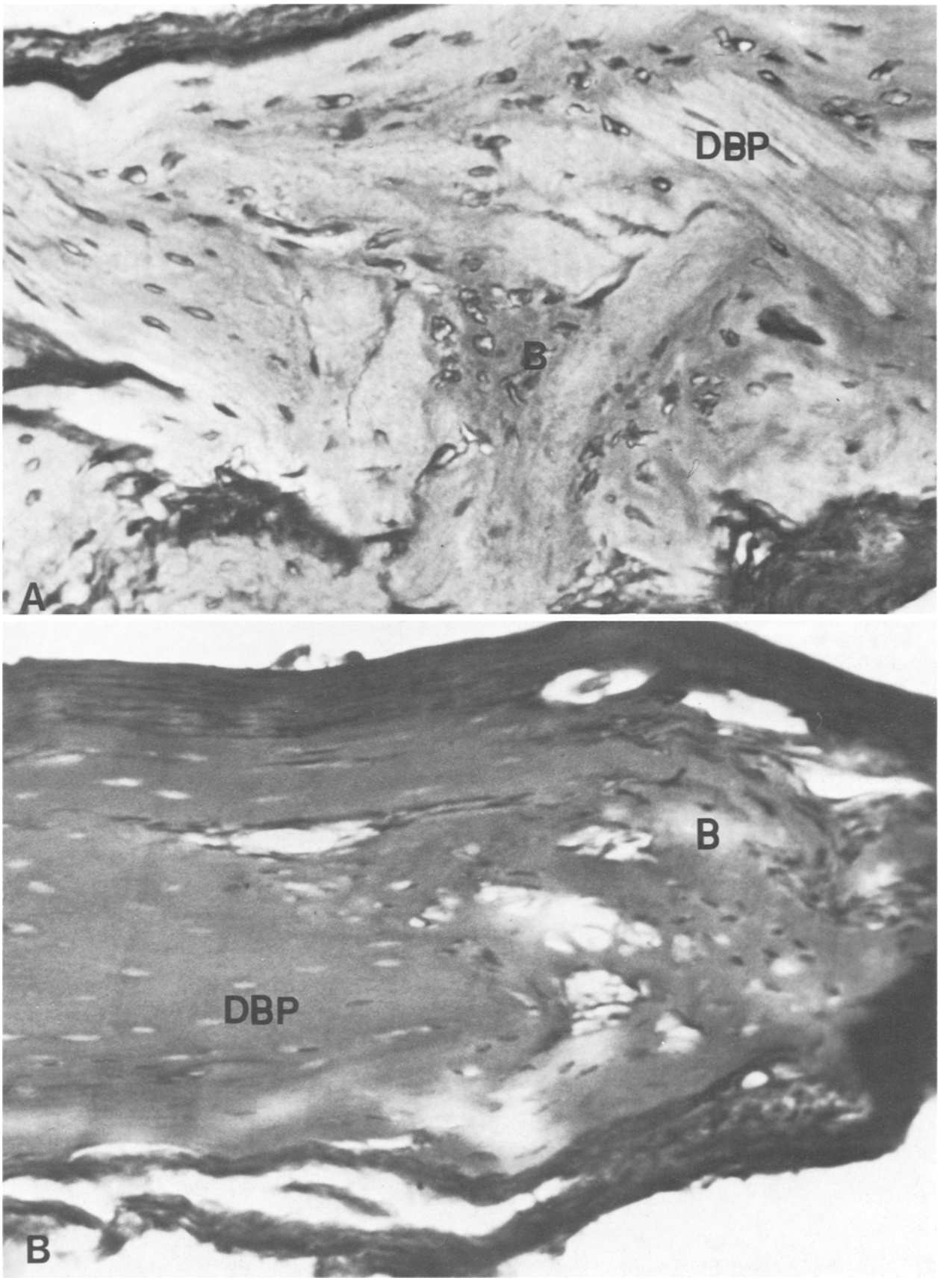

Fig. 3. Induced bone (*B*) surrounding demineralized bone powder (*DBP*) 2 weeks after implantation into cranial defects. **A** Particle size 75–250 μm. **B** Particle size 250–450 μm. Safranin-O. 640× magnification

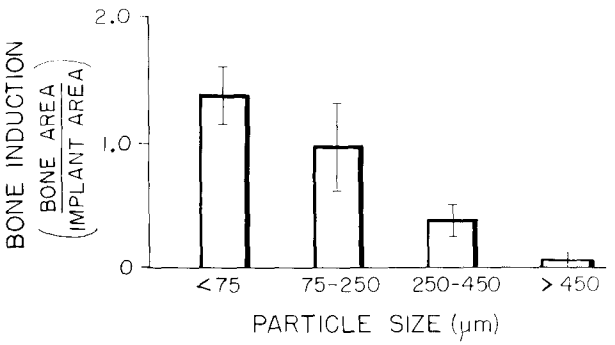

Fig. 4. Bone induction by equal amounts of demineralized bone powders of different particles size expressed as the ratio of new bone to implant area per field

Morphometric analysis.

Animals were sacrificed at intervals after implantation for histologic evaluation of the cranial defects. The specimens were fixed in neutral buffered 10% formalin, decalcified in 25% formic acid in 10% formalin, and embedded in paraffin. Sections (4 μm) were stained by hematoxylin and eosin, safranin-O, and van Gieson techniques. The following histological features were quantitated: (a) area of profiles of implanted particles as percent of the field, (b) area of cartilage or bone as percent of the field, and (c) number of multinucleated cells per field (450×). These measurements were made with a Ziess-Kontron MOP-3 digital image analyzer on 6 random fields from each of 4 implants per time point. The values were expressed as means of 24 fields ± SEM. Student's *t* test was used to determine significance.

Results

Cranial defects that had been rinsed with Ringer's solution ($N = 48$) filled in with fibrous tissue; they never healed with bone, even when followed for as long as 6 months.

All the defects that had been implanted with demineralized bone powder (DBP, 75–250 μm) ($N = 54$) were healed with bone by 2 weeks. Histologic analysis revealed that healing proceeded as a field transformation throughout the defect [9]. By day 5, a layer of host fibroblasts coated each particle of demineralized bone. On day 7, these cells had been transformed into chondrocytes. The amount of induced cartilage increased until day 10. Thereafter, the matrix became uniformly mineralized with adjacent neovascularization. Mast cells were seen in areas containing small blood vessels. By day 12, the cartilage was completely resorbed and replaced by bone. The amount of new bone and marrow increased until the implanted powder was solidly amalgamated within bone. Figure 1 demonstrates that the implanted DBP induced synchronous endochondral osteogenesis and that the implanted powder was not appreciably resorbed prior to bone induction nor during the experiment. The DBP remained amalgamated within the healed bone even at 6 months (Fig. 2).

Constant amounts, 10 mg, of demineralized bone powders of different particle sizes were implanted

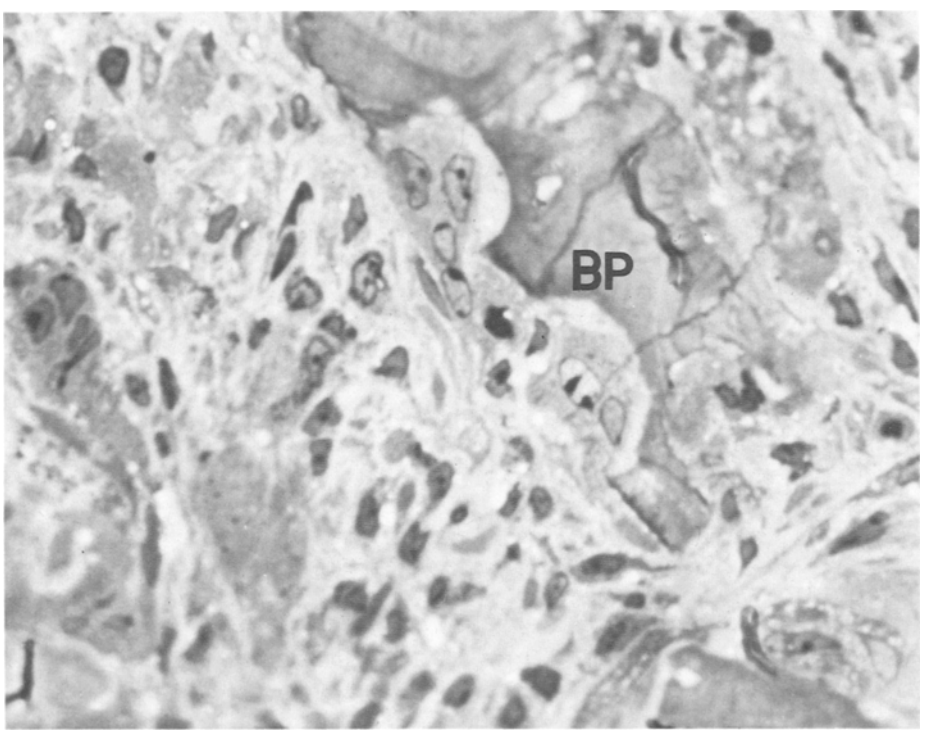

Fig. 5. Mineralized bone powder (*BP*) and host multinucleated cells 7 days after implantation into cranial defects. Toluidine blue. 640× magnification

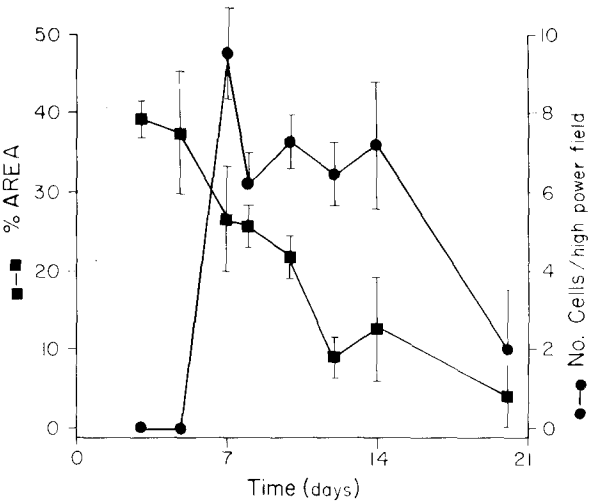

Fig. 6. Histomorphometric analysis of cranial defects: percent area of mineralized bone powder and presence of multinucleated cells

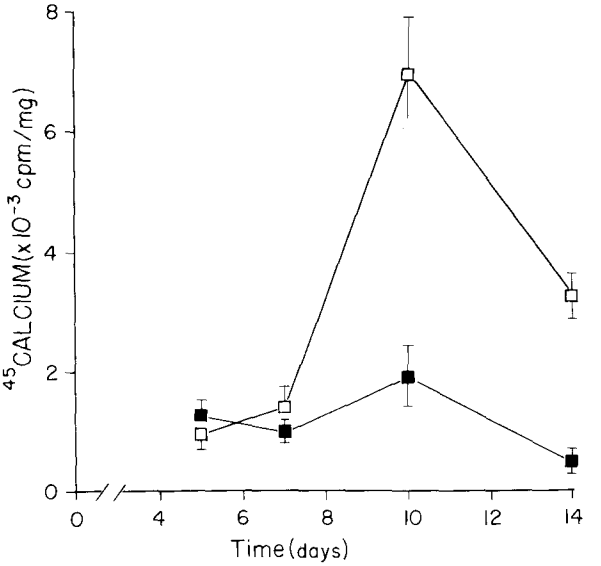

Fig. 7. Incorporation of ^{45}Ca into bone following implantation of demineralized bone powder (□) or mineralized bone powder (■) into cranial defects

into cranial defects. Figure 3 shows typical specimens 2 weeks following implantation. The smaller particles, presenting more surface area, induced more bone per field than did the larger particles (Fig. 4).

By contrast, bony healing did not occur when mineralized bone powder (BP) was implanted into defects ($N = 60$); only one showed evidence of new bone by 6 weeks. For the first 5 days after implantation, a layer of mononuclear cells surrounded each particle. After day 7, large multinucleated cells surrounded the bone powder (Fig. 5). Within 3 weeks, the implanted mineralized bone powder was resorbed from the site (Fig. 6).

Figure 7 represents the time course of calcification following implantation of either mineralized or demineralized bone powders, as judged by the incorporation of ^{45}Ca into the acid-soluble fraction of the implanted sites. The peak at 10 days corresponds to the first histological signs of mineralization of induced cartilage and bone. Calcium incorporation was greater in DBP-implanted sites than in BP-implanted sites ($P < 0.05$).

Implants of several preparations of hydroxyapatite (bone ash, deorganified bone, or commercially obtained hydroxyapatite) evoked the same response as did mineralized bone powder: the materials were surrounded by multinucleated cells and gradually resorbed from the sites. Implants of a mixture of DBP and ash (1:1 by weight), evaluated at 2 weeks, showed both responses: the pieces of ash were surrounded by multinucleated cells and the pieces of demineralized matrix were surrounded by induced cartilage and bone.

Discussion

The use of powdered forms of bone implants, presenting a large surface area to the recipient bed, has facilitated the evaluation of the fate and effects of demineralized and mineralized implants. Host responses to powders occur as highly synchronous field effects throughout the implant sites and are readily quantitated by histomorphometric and biochemical techniques.

These studies demonstrate the uncoupling of graft resorption and new bone formation. Mineral-containing implants undergo resorption whereas demineralized bone matrix induces osseous repair without appreciable resorption of the implant. Although these studies were performed in rats, they may offer insights into the physiology of bone grafting in craniofacial reconstruction [12] Conventional grafting techniques are based on the concept of creeping substitution, i.e. the graft is gradually vascularized and resorbed as new bone is synthesized [13]. The graft serves to provide immediate mass and stability and also to act as a scaffold for the ingrowth of new bone. With time, the grafts, particularly those in the craniofacial region, appear to melt away to an unpredictable degree. Further operative procedures are often required. This problem may be due to graft resorption exceeding bone ingrowth. To avoid excessive graft resorption, techniques using composites of autogenous marrow and fresh or banked bone have been developed [14]. Clinical studies are in progress to determine whether the use

of demineralized grafts will indeed bypass the obligatory resorptive phases and induce enduring bone.

This study shows that demineralized implants did not undergo appreciable resorption. These results with *devitalized* grafts are consistent with what is known about the resorption of *live* bone. It is presumed that for mineralized live bone to be resorbed, the mineral phase must be removed first so that the organic phase can be degraded by enzymes released from resorbing cells [15-17]. The mineral may be necessary for initiation of resorption of dead or living bone, perhaps by the attraction or attachment of resorbing cells to the mineralized substratum. Mundy et al. [18] have shown that resorbing bone releases factors chemotactic for peripheral monocytes. Others have shown that cell-matrix contact is required for bone resorption by peripheral monocytes [19] or by peritoneal macrophages [20].

The question remains whether the multinucleated cells described herein are related to osteoclasts or mononuclear phagocytes. Recently, Yakagi et al. [21] described similar cells surrounding EDTA-demineralized, NaB^3H_3-reduced bone particles within nylon pouches implanted subcutaneously in rats. Matrix resorption was determined by identification of radioactive collagenous peptides. Although their implants were demineralized, they failed to induce osteogenesis. This could be explained by major differences in our treatment procedures. It is interesting that when osteogenesis does not occur, for whatever reason, removal of particles takes place.

We propose that the model of intracranial implantation of demineralized and mineralized bone powders in rats may be useful for studying the life cycle of the cells involved in bone synthesis or resorption as well as hormonal and drug effects on these processes.

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