Chapter 15

COMPOSITE BONE GRAFTS

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THE TERM *composite bone graft* refers to a graft that contains both bone and marrow. Since the pioneering work of Burwell,[1, 2] it has come to have a more specific meaning. Composite bone grafts (CG) are implants of allogeneic/xenogeneic bone with autologous marrow. This is a very special situation with important implications for clinical orthopedics. Whereas allogenic and xenogenic bone grafts per se can provide a certain degree of mechanical support, providing they are large enough to withstand destruction until osteogenic elements "creep in" from the neighboring host tissues and gradually replace the graft, they are foreign bodies that are ultimately destroyed via the immunological responsiveness of the host. If, on the other hand, allogeneic[3, 7] and xenogenic bone grafts[8, 9] are washed free of native marrow and are then impregnated with host-derived autologous marrow, they escape immunologic recognition mechanisms and become as biologically compatible and successful as bone autografts.

Deproteinized xenogeneic bone substrates (Keil Bone) are also as satisfactory components of composite grafts as native allogeneic bone; work with Keil bone-autologous marrow grafts has proceeded beyond the basic science laboratory.[8, 9] They have been implanted in large, difficult-to-heal osseous defects in humans with good effect.[10, 11] The development of studies in laboratory animals, however, has been critically important for our understanding of the mechanisms involved. Earlier, there was little doubt that the marrow cells were a most important component in these grafts. Composite grafts failed, so to speak, when the osseous component was infiltrated with killed marrow cells. As novel and important as Burwell's studies were, the concept that marrow contained putative osteogenic cells had not been totally undeveloped. From the earlier literature, particularly the well-constructed studies of Danis,[12] we knew that marrow cells were competent to form bone in a diverse number of anatomical sites, e.g. under the kidney capsule, in spleen, skin, muscle, epicranium, liver, and in the anterior chamber of the eye. We also knew from the pioneering studies of Bloom[13] that, in avian species, the stromal elements of marrow were most likely the origin of cells called preosteoblasts or reticular cells. It remained for Freidenstein[14, 15] to demonstrate most convincingly that marrow cells in culture contained fibroblast colony forming units (FCFUs) that express an osteogenic potential when implanted in vivo in Millipore®

diffusion chambers. In addition, we now know from marrow extirpation-recovery studies[16, 17, 18] that osteogenic precursor cells residing in the linings of intracortical bone spaces can grow out into the medullary cavities before repopulation of the hemopoietic cell stock, indicating that the marrow stroma organizes the microenvironmental niches that are attractive to colonizing hemopoietic cells.

One problem arising from CG studies heretofore has been that the grafts were implanted intramuscularly; it could not be ascertained, therefore, if the populations of respondent osteogenic cells were drawn in equal or unequal proportions from the marrow or from the pluripotent cells in the graft bed. Even grafts with dead marrow cells, for instance, show small amounts of new bone formation, but we do not know precisely the degree of exclusion of participant mesenchymal cells in the graft bed that are mobilized when challenged with inductive matrices.[19] In the same way, it has not been possible to exclude the possibility that the bony component of the CG influences the osteogenic response, perhaps via release of a bone morphogenetic protein. While the answers to these questions relate to the mechanism that governs the behavior of the grafts, they do not speak to an additional problem, e.g. how one might optimize the CG. Is there, for instance, a favorable ratio of marrow cells to bone? How few marrow cells are required to make CGs perform as well as autografts? Similarly, if the prospective recipient of a CG has too little available bone and marrow, might it be possible to raise larger populations of these cells in culture (with mitogens) and expect them to have preserved their osteogenic potential?

Materials and Methods

General Considerations: For all of these experiments, we used domestic rabbits as the source of bone and marrow. Under serotol anesthesia (30 mg/kg I.V.), the animals were shaved over the left hip and abdomen. These areas were then swabbed with Betadine® and draped prior to operation, which was carried out under sterile conditions. The crest of the ilium was exposed, freed of soft tissues, and a bone biopsy (about 1 × 1 cm) was taken. After the outer cortices of the biopsy specimen were trimmed away to exclude as much cartilage as possible, the tissue was cut into several fragments of cancellous bone, each a few millimeters thick. These bone chips with their marrow were used intact as autografts.

In the preparation of composite grafts, the marrow from the cancellous (donor) bone chips was washed away with a jet of 0.9% saline from a syringe until the bone appeared white. While this procedure failed to effectively remove the (monolayer of) spindle-shaped, potentially osteogenic cells directly adherent to the bone, we have demonstrated that these cells do not appear to play an important role in the success of CGs. The washed bone fragments were then impregnated with fresh red marrow

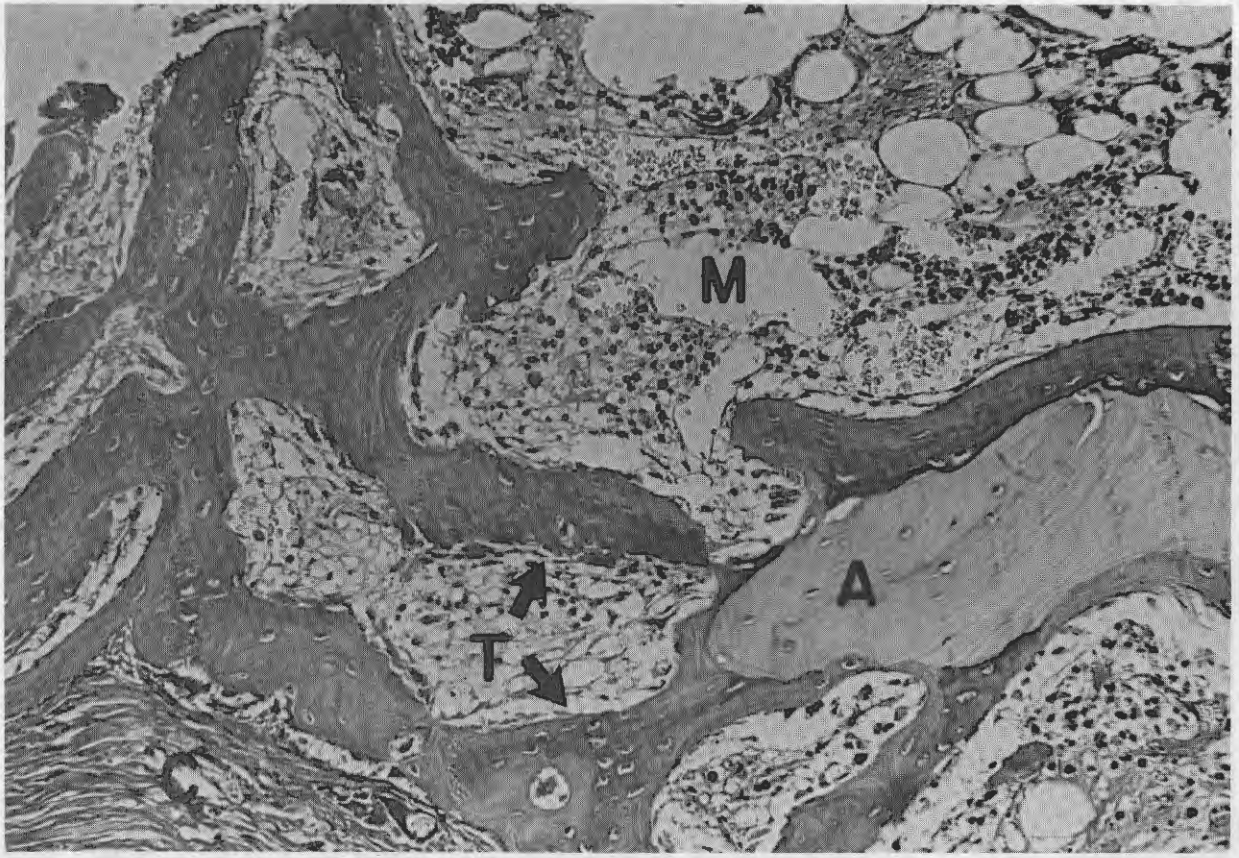

Figure 15-1. Photomicrograph of new bone formation (T) in a composite graft of allogeneic bone (A) and autologous marrow (M) five weeks after transplantation to the rectus muscle (C) of a rabbit. Hematoxylin and eosin.

obtained from the femurs of the recipient rabbits as described by Burwell[1, 2] and inserted into the graft site. Figure 15-1 shows the behavior of the graft tissue five weeks after implantation. There was much new bone formation.

Osteogenic Role of Marrow Cells: In an attempt to determine if the integrity of CGs was such that they could support new bone formation without contribution of osteogenic cells from the graft bed, we conducted two different kinds of experiments.

In the first,[3] we isolated CGs of allogeneic bone and autologous marrow in Millipore chambers (0.45 μm pore size). The marrow cells (donor origin) in these grafts had been obtained from the animals labeled with tritiated thymidine (^{3}HTdr), so that we would be able to trace the origin of the newly formed bone cells within the grafts with some certainty. Autoradiographs of the "graft materials" indicated that 60 percent of the marrow reticulocytes (osteoprogenitor cells) had been labeled when thymidine was administered in three injections at eight hour intervals a day before biopsy and grafting. Three, five, and seven weeks after implantation, the chambers were recovered and subjected to histologic-autoradiographic investigation to determine (1) the degree to which the marrow cells had participated in new bone formation, e.g. the appearance of labeled osteocytes in grafts, and (2) whether CGs per se were capable of producing a diffusible osteoinductive material that could pass through the filters and cause the primitive mesenchymal cells in the adjacent graft bed to differentiate into osteogenic elements. The autoradiographs were prepared by dipping histologic sections into liquid NTB-2 emulsion; the preparations were allowed to expose in a freezer, and they were subsequently developed and stained through the emulsion with hematoxylin and eosin.

In the second experiment, a variant of the cell labeling technique was attempted. CGs were fashioned of (A) autologous allogeneic bone and thymidine-labeled marrow cells for implantation into "cold" host rabbits or (B) "cold" bone-marrow grafts were implanted into hosts that had been preinjected with ^{3}HTdr three times in a twenty-four hour period (intervals of eight hours) to label the pluripotent mesenchymal cells in the implant site. The grafts were recovered after three, five, and seven weeks and examined autoradiographically to determine if, in Study A, the initially labeled marrow cell components provided the osteoblasts and if, in Study B, the mesenchymal cells in the graft bed contributed significant numbers of osteoblasts.

Composite Graft Optimization

Variation in Marrow Cell Census: In these studies,[4] CGs were prepared from two strains of inbred rabbits obtained from the Bar Harbor

Laboratories. Strain III rabbits had undergone 18 generations of sib-mating; Strain B, 22-25 generations. The Strain III rabbits were always used as the recipients (marrow donors) while Strain B rabbits provided the bony fraction of the grafts. In these studies, the CGs contained 39.6 to 44.0 mg washed (autogenous or allogenic) bone chips impregnated with 10,000 or 50,000 viable marrow cells that had been grown in culture for ninety-six hours with M-199 containing 100 units of penicillin and 50 units streptomycin/ml. Replication of these cells in vitro was demonstrated by their increase in DNA concentration following PHA stimulation.[4] The object of these studies was to determine the "efficiency" with which (different populations of) marrow cells could sustain new bone formation. We also asked whether it might be possible to improve the osteoinductive response of suboptimal populations of putative osteogenic cells in the marrow component of the grafts by stimulating these cells to proliferative in vivo, e.g. by treating the host rabbits with a mitogen immediately after grafting (6.25 mg bactophytohemagglutinin via an ear vein four times at fifteen minute intervals) and then on an alternate day schedule (25 mg I.P.) for two to five weeks. Here we were exploiting the mitogenic capacity of the lectin PHA, since it has been shown many times [20-23] that PHA will not result in immune suppression of humoral antibody formation when it is administered at or after the time of antigenic challenge.

As an adjunct to these studies, we also explored the osteogenic capacity of composite grafts by fashioning grafts with varying proportions of autologous marrow cells (500-2200/mg bone) and allogeneic spleen cells (100% marrow or 100% splenocytes; 50 : 50 marrow-splenocytes; 33 : 66 marrow-splenocytes). The aim of this study was again to demonstrate that it was the marrow cells that were responsible for the success of composite grafts. One would have had to employ autologous populations of splenocytes if the purpose had been to demonstrate that the spleen contained putative osteogenic elements (inducible osteoprogenitor cells of Friedenstein) in sufficient quantity to support bone formation. This remains a question for future investigation. In all of these studies, as well, the grafts were inserted into a well-vascularized pocket in the rectus muscle, and they were recovered after five weeks.

Variation in Bone Quality: While the foregoing sections describe our methods for the preparation of the routine CGs, we have used other allogeneic osseous substrates that were prepared in ways intended to reduce their antigenicity, e.g. freeze-thawing, freeze-drying, and surface demineralization (0.6*N* HCl). Surface demineralization particularly has the effect of producing a matrix that promotes chondrogenesis/osteogenesis in intramuscular sites.[2, 18, 24-27] The other procedures were designed to produce a matrix that could simply be expected to survive transplantation owing to preservation of a diffusible bone morphogenetic

protein (glycoprotein). As additional controls we implanted CGS constructed of (a) fresh allogeneic bone with dead autologous or allogeneic marrow cells and (b) fresh allogeneic bone with admixtures of fresh autologous marrow cells and fresh allogeneic splenocytes (see below). In all of these studies,[5] the grafts were implanted into a well-vascularized rectus muscle of recipient rabbits and they were recovered after three and five weeks.

Quantitation of Graft Behavior: In all of these studies, our estimates of osteogenicity were based on the percent of graft bone surfaces covered by newly formed bone. This was accomplished hy point counting using an ocular reticule.

Results

Osteogenic Role of Marrow: The various approaches we[13] have used in these studies confirmed previous observations and inferential evidence that marrow contains cellular elements capable of becoming osteoblasts.

Millipore Chamber Experiments: When composite grafts with ^{3}HTdr-labeled marrow cells were loaded into Millipore chambers and, thereby, isolated from other cells in the body — some of which could be hematogenously derived migratory elements with osteoprogenitor cell properties — two types of tissues differentiated. At three weeks, cartilage grading to osteochondroid was observed on the surfaces of the allogeneic bone component. The cartilaginous tissue stained irregularly or not at all with Saffranin-O and Azure II, suggesting that it might he deficient in glycosaminoglycans. At five weeks postgrafting, the chambers contained ossicles of new bone with a central marrow core. The autoradiographs indicated that the new bone osteocytes were labeled (^{3}HTdr) and that they therefore had been derived from the marrow elements.

Composite Grafts in ^{3}HTdr-Labeled Hosts: Based on autoradiographic evidence, we observed that CGs implanted in hosts that had been prelabeled with ^{3}HTdr did form bone, but the bone did not contain labeled osteoblasts and osteocytes. On the other hand, when "free" CGs bearing marrow cells from a donor that had been prelabeled with ^{3}HTdr were implanted intramuscularly, the graft showed many labeled osteohlasts and osteocytes (Fig. 15-2), as well as labeled osteoclasts (Fig. 15-3). This suggested that when allogeneic bone is infiltrated with autologous marrow, the marrow (B-cells) effectively isolates the graft bone, and probably protects or shields the bone allograft, so that it is either not recognized by the immune defenses of the host or it is made inaccessible to effector cytolytic T-cells and to the putative osteoprogenitor cells in the host bed. We now recognize that the presence of labeled nuclei in osteoclasts indicates formation from monocytic-macrophagic components[28, 29] and these elements were doubtless included as original components of the marrow fraction.

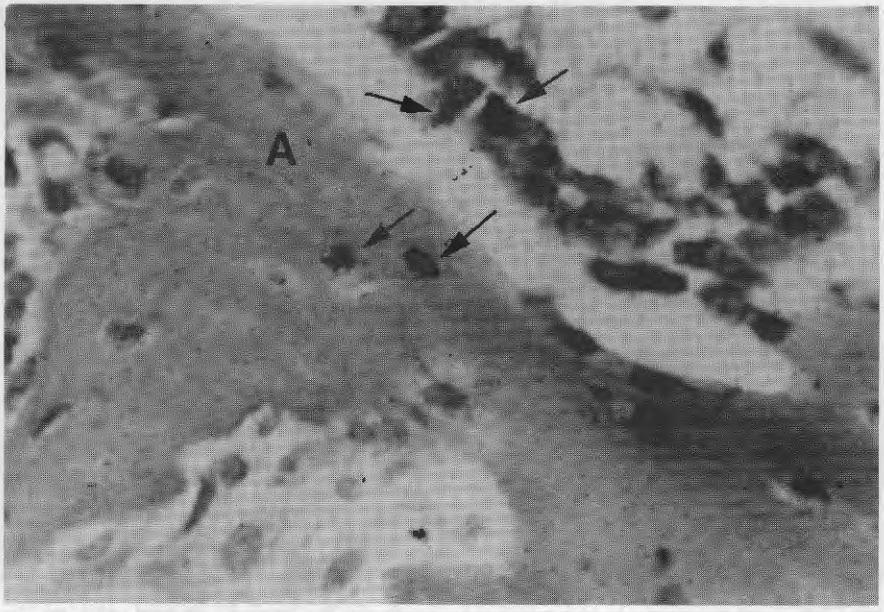

Figure 15-2. Autoradiograph of a composite graft of allogeneic bone and tritiated thymidine labeled autologous marrow cells five weeks after transplantation to the rectus muscle of a rabbit. Osteoblasts with labeled nuclei (arrows) are seen adjacent to the newly forming bone (A), which contains several labeled osteocytes (arrows), indicating an origin from cells in marrow. Hematoxylin and eosin.

Optimization of Composite Grafts: In an extended series of investigations,[5] we have asked whether it might be possible to improve the osteoinductive response of CGs. As noted above, the devices we employed were twofold. First, we altered the numbers of autologous marrow cells used to infiltrate a standardized allogeneic cancellous bone implant. Second, we attempted to employ CGs in which the bone matrices were (by weight) altered by freeze-thawing (killed), freeze drying, or surface demineralization. Here we tested the surviveability and bone inductive capacity of the matrices upon the marrow cells.

Marrow Cell Numbers: The critical evidence that there is an optimal ratio of bone to marrow cells was developed in studies[4] using inbred strains of rabbits, and these are summarized in Table 15-I. The data strongly supported the concept that the success of a composite graft, as measured by its ability to provoke a level of bone formation equal to that of a bone-marrow autograft, is dependent upon its complement of marrow cells. The response could be shown not to be due to a significant contribution of osteoprogenitor cells from cells in the graft bed. Five weeks after grafting, CGs consisting of 39 mg allogeneic bone and 10,000 viable autologous marrow cells (raised in culture for 96 hours) showed new bone formation on only 10 percent of the graft bone surfaces versus 30 percent

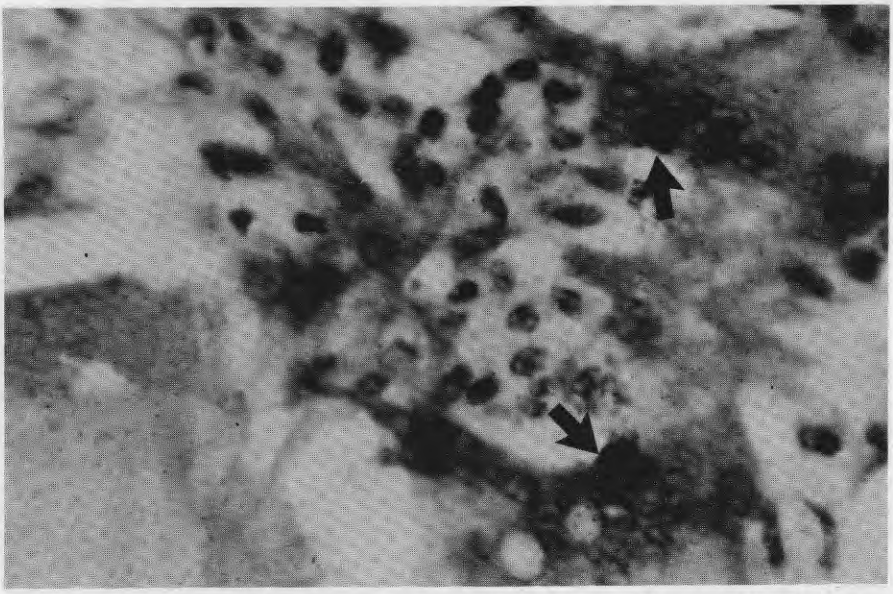

Figure 15-3. Autoradiograph of a composite graft of allogeneic bone and tritiated thymidine labeled autologous marrow cells five weeks after transplantation to the rectus muscle of a rabbit. Two osteoclasts with labeled nuclei (arrows) are seen in the vicinity of the graft, indicating an origin from monocytic macrophagic elements in the marrow. Hematoxylin and eosin.

of surfaces of new bone formation when the grafts contained 50,000 cells (autograft response). Grafts with dead marrow cells (freeze-thawing), on the other hand, were completely noninductive and they were undergoing resorption by large multinucleated giant cells (?macrophages) at the end of five weeks (Fig. 15-4). As interesting as these results were, we then determined that it was possible to markedly increase the performance of grafts with initially suboptimal numbers of marrow cells by administering PHA to the host rabbits. Table 15-I shows that the course of PHA injections improved the osteoinductive capacity of grafts with 10,000 marrow cells so that a more "normal" percent of graft surface was undergoing bone formation (autografts). However, when PHA was injected into animals bearing composite grafts with 50,000 cells, an already optimal number for the weight of bone used, no improvement in graft performance was noted.

These findings have been confirmed in other studies. Figure 15-5 shows that intramuscular-sited CGs with 1000-2000 cells/mg produce as much new bone as (a) native intact bone-marrow autografts, (b) washed (marrow free) autologous bone, and (c) reconstituted autografts (marrow washed out and reintroduced). Suboptimal responses were realized when the implants contained as few as 500 cells/mg bone. The importance of mar-

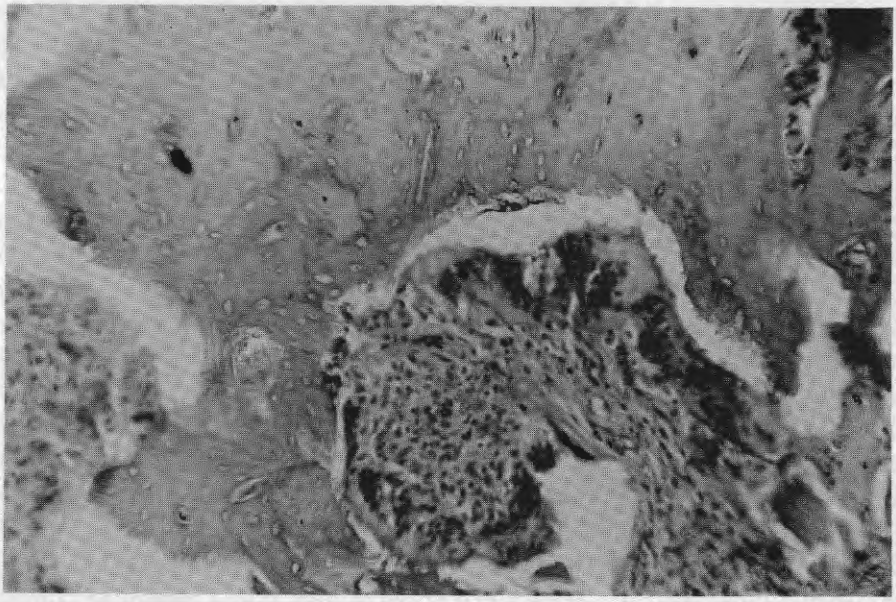

Figure 15-4. Photomicrograph of a graft of allogeneic bone showing resorptive giant cells (macrophagic) on its surfaces five weeks after transplantation to the rectus muscle of a rabbit. These grafts rarely show foci of new bone formation.

TABLE 15-I

PERCENT OSTEOINDUCTIVE SURFACE IN BONE GRAFTS

	DUTCH RABBITS		STRAIN III HOST RABBITS			
				Composite Grafts		
Group	Autografts	Composite grafts	Autografts[a]	Strain B bone + dead marrow cells	Strain B bone[b] + 10,000 III marrow cells[d]	Strain B bone[c] + 50,000 III marrow cells
-PHA	33.3 ± 9.8 (5)	34.9 ± 2.5 (4)	23.5 ± 3.7 (10)	0 (12)	9.68 ± 3.9 (5)	29.3 ± 4.6 (5)
+PHA	–	–	23.0 ± 4.9 (12)	–	29.3 ± 5.7 (6)	21.9 ± 5.9 (5)
					$p < 0.02$	p =N.S.

[a] Weight of bone-marrow autograft = 100 mg.
[b] Weight of bone = 39.6 ± 6.7 mg.
[c] Weight of bone = 44.0 ± 9.0 mg.
[d] Cultured for 96 hr prior to use in the composite grafts.

row cells is again stressed when one considers the low "background" level of bone formation that is found when allogeneic matrices are implanted without marrow or with killed marrow. It is unfortunate that these experiments did not include CGs with more than 2200 cells/mg bone, but the method of quantification of the osteogenic responses was such that we were already close (80-90%) to the theoretical maximum of surfaces occupied with new bone. Any extension of these studies would most likely have to take into account the total volume of new bone produced and not rely solely upon quantification of surfaces of the implant engaged in new bone formation. However, within the limitations of the present system, we were able to demonstrate that CGs consisting of allogeneic bone and variable admixtures of freshly isolated autologous marrow cells and allogeneic splenocytes produce as much new bone as the marrow cell numbers allow. Figure 15-6 shows that while 80-90 percent of graft surfaces were covered with new bone when the composite grafts contained 100 percent marrow cells, the progressive addition of splenocytes (50%M : 50%S, 333% : 66%S, and 100%S) reduced the osteogenic capacity of the grafts (Figs. 15-7 and 15-8) until "background" levels were approached, e.g. a response comparable to that seen in grafts of allogeneic bone with or without dead marrow or in implants of allogeneic bone and allogeneic marrow (Fig. 15-4).

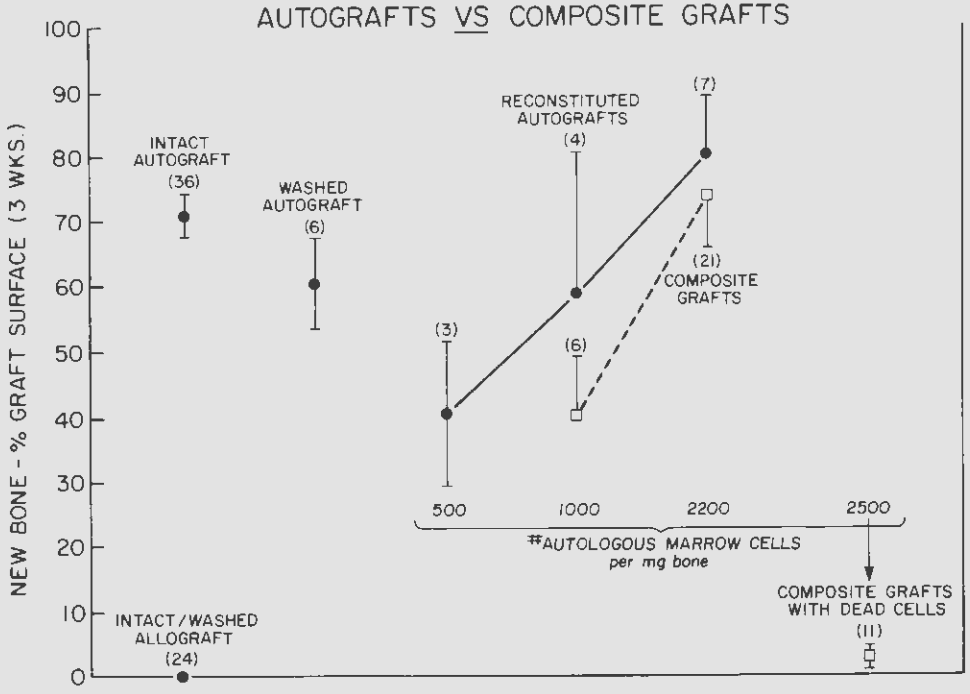

Figure 15-5. Plots of the osteogenic potential of different kinds of bone grafts three weeks after transplantation to the rectus muscle of rabbits.

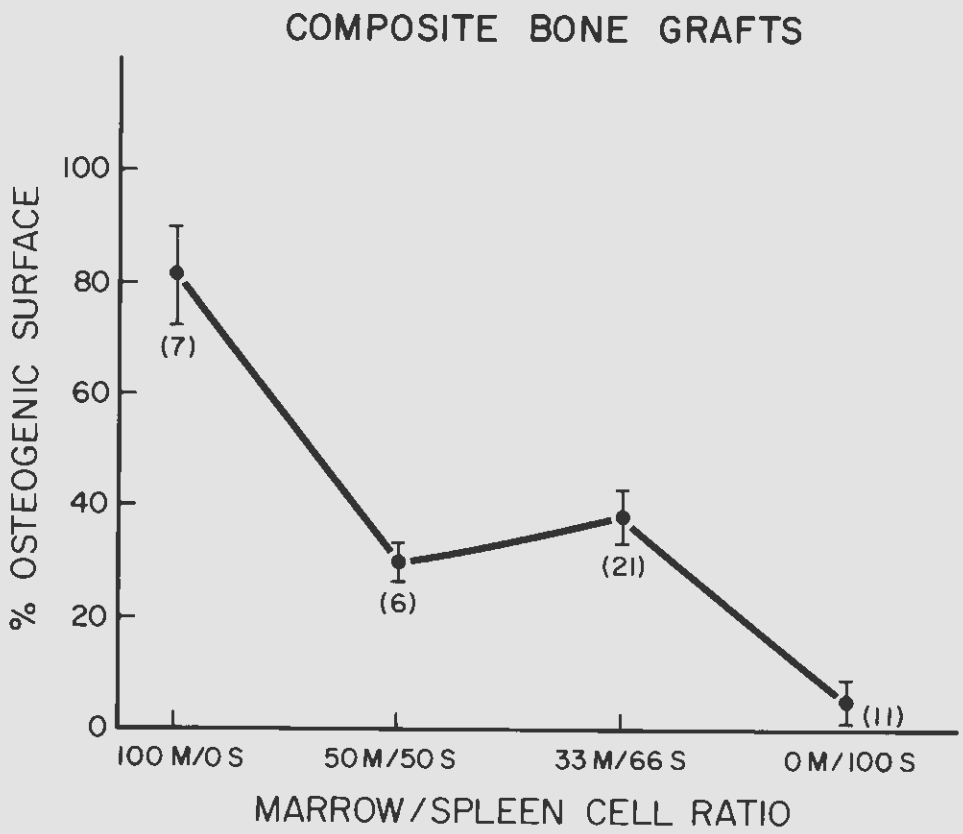

Figure 15-6. A plot of the osteogenic potential of composite grafts fashioned from allogeneic bone and varying proportions of autologous marrow and allogeneic splenocytes. The amount of new bone formed in these grafts is related to the percentage of the marrow cell component.

Since there appeared to be an "upper limit" to an effective marrow cell number, we turned our attention to the interrelationships between marrow cells and the bony substrate. Would a change in bone quality further improve the performance of composite grafts?

Nature of the Bony Component of Composite Grafts: We have already alluded to the fact that few graft materials — whether autologous, allogeneic, xenogeneic, and even demineralized matrices — survive transplantation, although they may, for a time, seem to support osteogenesis. The limit imposed may be the numbers of osteoprogenitor cell elements in their immediate vicinity and, in the case of demineralized matrices, whether or not they have been processed in such a way as to preserve their glycoproteinlike bone morphogenetic protein. The studies we report herein[5] compare the performance of CGs in generating new osseous tissue

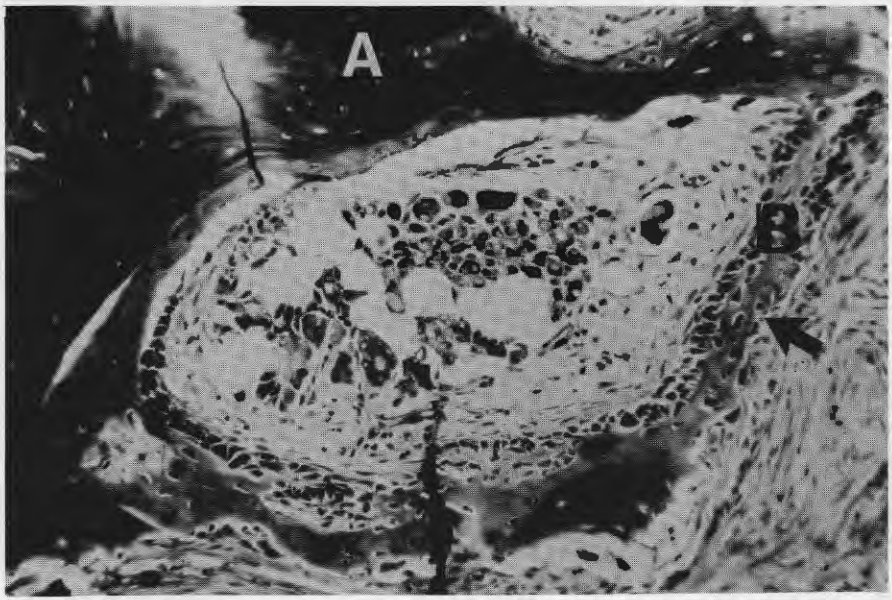

Figure 15-7. Photomicrograph showing newly forming bone trabeculae (B) in a composite graft of allogeneic bone (A) and a 50:50 mixture of autologous marrow cells and allogeneic splenocytes. The graft was harvested five weeks after transplantation to the rectus muscle of a rabbit. There are some newly forming trabeculae of bone bordered by a row of active osteoblasts (arrow) and macrophages reside within the ossicle. Hematoxylin, eosin, and Axure II.

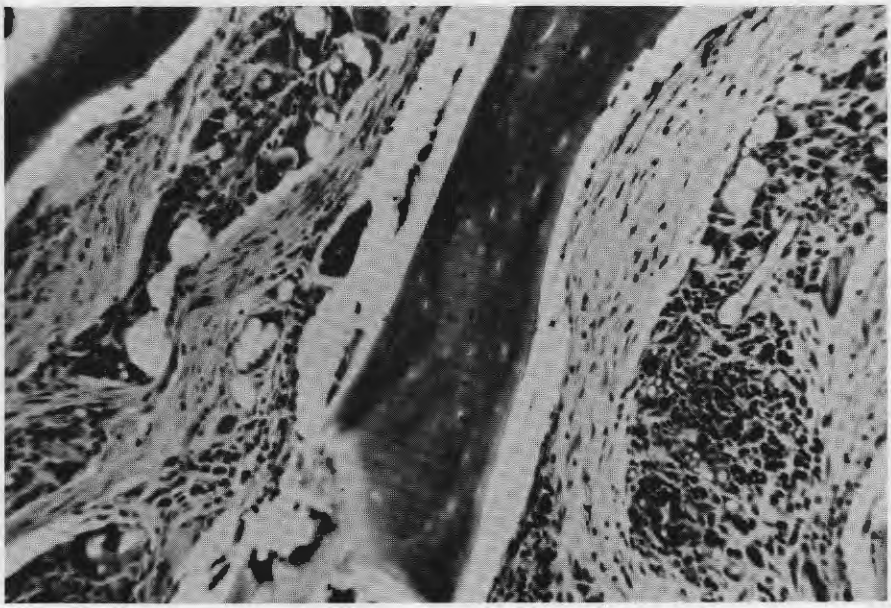

Figure 15-8. Photomicrograph showing peritrabecular fibrosis around a composite graft of allogeneic bone and allogeneic splenocytes (*see* Fig. 15-6) five weeks after transplantation to the rectus muscle of a rabbit.

when the bony component consists of a marginally or fully active inductive substrate.

Figure 15-9 indicates that the nature of the bone matrix does affect the performance of CGs containing an adequate population of autologous marrow cells (100/mg). Whereas no important osteogenic response occurred when various types of allogeneic matrices were combined with allogeneic marrow, CGs consisting of surface demineralized bone (0.6*N* HCl) and freeze-dried bone seemed to be the equal of some unaltered allogeneic matrices combined with 1000 or 2200 cells/mg. In these preliminary studies, demineralized bone particularly stands out since its complement of 1000 cells produced as much new bone as fresh bone impregnated with 2200 cells/mg. The study comprised too few grafts to really establish this point firmly, but Urist's laboratory has greatly expanded and confirmed this phase of the work. They report[29] that CGs with demineralized bone are more successful than autologous grafts within times short after grafting (one to two weeks). One may infer from these experiences that the possibility of preparing biocompatible and actively osteoinductive

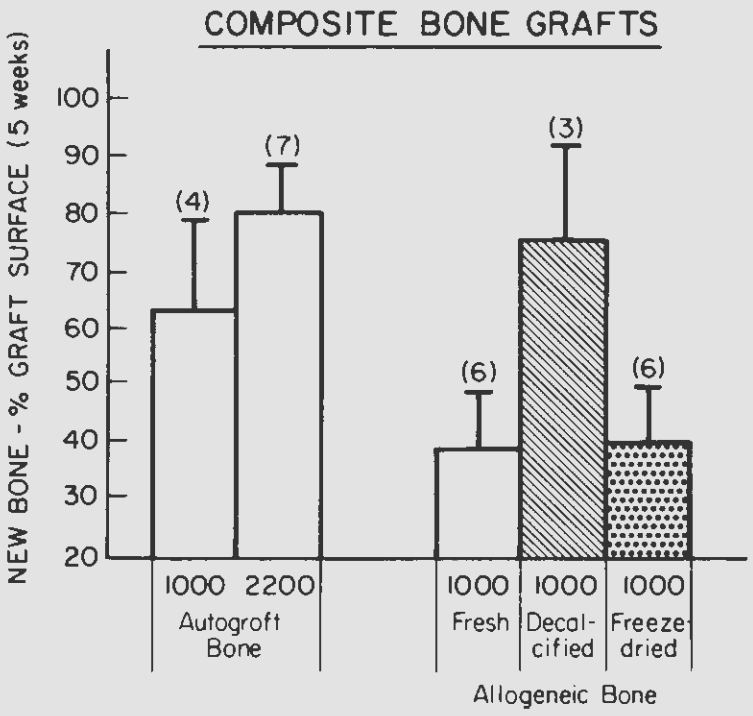

Figure 15-9. Graph comparing the performance of composite grafts of Autologous Bone-Marrow with composite grafts of specially prepared matrices.

matrices for use in composite grafts signals a way in which the technique can be enhanced for clinical application.

Comment

In this chapter, we have cited a number of experiments that have exploited knowledge that bone marrow contains a resident population of cells with stem cell and osteogeneic potential. It is clear that some of these cells, probably those referred to in the early literature as preosteoblasts or reticulocytes (stromal cells), are capable of giving rise to progeny that can become osteoblastic in the absence of any apparent inductive substance (determined osteogenic precursor cells of Friedenstein[14, 15]). The marrow contains these precursor cells probably as a fixed fraction.

It may be inferred from the studies reported herein that under normal circumstances only a fraction of the osteoprogenitor cell pool is activated, that the osteogenic response can be heightened by altering (surface demineralization) the nature of the bone matrix component so that its inductive capacity can be expressed. This hypothesis is perhaps made more attractive by our observations that the success of composite grafts is entirely the province of the contained marrow cells; the contribution of osteoprogenitor cells from the complement of mesenchymal cells in the soft tissues at the graft site seemed to be inconsequential. Practical grafts combining Kiel bone (deproteinized and thereby lacking in osteoinductive properties) and autologous marrow have already seen clinical use,[11] but this is doubtless a different situation. The possibility of preparing actively osteoinductive matrices for use in CGs signals a manner in which the technique can be enhanced for clinical use in the future.

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