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Survival of Osteocompetent Marrow Cells *in Vitro* and the Effect of PHA-Stimulation on Osteoinduction in Composite Bone Grafts[1] (38673)

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Fresh calcified allogeneic bone grafts placed in a heterotopic site such as muscle do not long survive, or they have a temporally limited capacity to serve as an osteoinductive substrate (1). Allograft success can be improved if the implants are partially decalcified in 0.6 *N* HCl (1, 2) and/or incubated in a general metabolic inhibitor such as iodoacetate (3, 4), or if they are admixed with autologous marrow to form a composite graft (5–8). Composite grafts with xenograft bone are also inductive (9, 10). On the basis of these and other biochemical evidences, Urist (4) proposed that bone and cartilage induction is due to the action of a transmissible bone morphogenetic protein (BMP). His laboratory has since characterized BMP as an insoluble noncollagenous protein or polypeptide, or as part of a protein firmly bound to collagen (4, 11).

In a heterotopic graft, BMP is the transmissible factor thought to induce undifferentiated perivascular connective tissue cells in the host bed to become osteoblastic or chondroblastic. The mechanism may be similar to composite grafts in which fresh autologous marrow is infiltrated into allogeneic trabecular bone after its highly antigenic red marrow has been carefully washed away. The success of the composite graft could be due to the fact that the autologous marrow provides large numbers of inductible perivascular connective tissue cells in the immediate BMP environment (10, 24) and perhaps because the accompanying B-cells shield the bone allograft so that it is not either recognized by the immune defenses of the host or it is made inaccessible to effector cytolytic T-cells. The data on osteoinduction also suggests that the efficiency of the process in composite grafts should vary with the number of presumptive target cells for BMP, and that it might be possible to improve the osteogenic capacity of composite grafts if we had some measure of the number of marrow cells per unit mass of allogeneic bone required to sustain good growth. We have investigated this problem in the present study. We have also asked whether it might be possible to improve the osteoinductive response of suboptimal populations of inductive cells in the marrow component of a composite graft by stimulating these cells to proliferate with the mitogen PHA (12).

Materials and Methods. Graft preparation. Outbred Dutch rabbits and inbred Strains B and III rabbits (Jackson Memorial Laboratory, Bar Harbor, Maine, 2–3 kg) were used in this study. In experiments with Dutch rabbits, cancellous bone was obtained from the iliac crests under sterile conditions. The autografts with intact marrow (wt = 100 mg) were a few mm thick. The composite marrow autografts—bone allografts were prepared by flushing the marrow out of some bone chips with a jet of saline from a syringe with a fine needle until the bone appeared white. This bone was then impregnated with fresh red marrow taken from the femurs of the recipient rabbit.

In the experiments with inbred rabbits (Strain III—18 generations of sibmating; Strain B—22–25 generations), the Strain III rabbits were always used as the recipients. They were grafted autologously with intact bone-marrow preparations from other Strain III donors, and saline washed donor Strain B bone was used to make the composite grafts which were packed with marrow cells from Strain III rabbits. The Strain III marrow was aspirated into a heparinized syringe asceptically from the tibial shaft through a surgical osteotomy made with a micro-Stryker saw. Each ml

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of marrow was suspended in 5–10 ml 3% dextran in saline, and allowed to stand for 1 hr. Cell concentrations were estimated with a hemocytometer.

The composite grafts were of three types: (a) 100 mg washed allogeneic cancellous bone chips infiltrated with marrow cells that had been killed by freezing in liquid nitrogen and thawing 3×; (b) 44 ± 10 mg washed allogeneic cancellous bone chips (Strain B) infiltrated with 50,000 viable marrow cells (Strain III) in TC-199 media; and (c) 38 ± 9 mg washed allogeneic trabecular bone chips (Strain B) infiltrated with 10,000 viable marrow cells (Strain III) which had been grown in culture for 96 hr with TC-199 containing 100 units penicillin and 50 mg streptomycin/ml. No attempt was made to estimate the proportions of marrow cells and undifferentiated reticular cells in each of the marrow samples that might serve as the osteoinductive elements for the grafts.

Graft implantation. At the time of surgery, all autologous and composite bone grafts were kept in chilled TC-199 medium. The animals were anesthetized lightly with sodium pentobarbital and the bellies of the recipient rabbits were shaved, scrubbed with betadine, and draped for surgery using aseptic technique. Anesthesia was maintained with ether. A midline incision was made, and six autografts were inserted into pockets made in the left rectus muscle. Marrow-free allogeneic grafts as a control, or composite grafts were implanted on the right side. Each pocket was then closed with 1–2 sutures of 4–0 polyethylene, and the midline incision was closed with 4–0 silk and sprayed with aeroplast. 30,000 units of penicillin was given im postoperatively, and this dose was repeated 24 hr later. Grafting was always done between 0800 and 1200 hr.

PHA Administration in vivo. Following the surgical procedures, the rabbits that had received composite grafts with 10,000 and 50,000 marrow cells were given four injections of 6.25 mg Bacto-Phytohemagglutinin-P (PHA) *via* the ear vein at 15 minute intervals. The rabbits with grafts containing 50,000 marrow cells were given an additional 25 mg ip every other day at 1200 hr until the time of sacrifice 5 wk later. PHA was given only during the initial 2 postsurgical wk to the animals grafted with 10,000 marrow cells.

Autopsy procedures. Five weeks after surgery, the animals were sacrificed by an overdose of pentobarbital. The graft sites were excised *en block*, fixed in 10% neutral formalin for 48 hr, and X-rayed to visualize the location of each of the implants. The implants were then decalcified in formalin-EDTA for 2 wk, and then were submitted for routine histologic processing. Serial sections were cut at 5 μm, and stained with hematoxylin, eosin and azure II. Marrow cells that were not employed in processing for the composite grafts were fixed in 1:3 acetic acid and methanol, centrifuged, resuspended and smeared on slides. The slides were stained with Giemsa.

Graft quantitation for new bone formation. The outcome of the grafting procedures on promoting bone formation were quantitated by point counting technique using an ocular grid overlay at 200× magnification. We counted the total number of horizontal lines in the ocular grid (11/microscopic field) that intersected non-inductive allogeneic bone surfaces and the surfaces of newly-induced bone trabeculae. The relative osteoinductive surface was calculated by dividing the number of intersects with newly induced bone tissue by the total number of surface intersects. In practice, we considered that both surfaces of a newly induced bone trabeculum were osteogenic even though the connective tissue-bone interface rarely showed osteogenic activity. It seemed practical to follow this procedure to avoid underestimating the amount of new bone present in the grafts. It was not feasible to assay the inductive capacity of the grafts by comparing the microscopic volume ratios of new/old bone because some implants included more cortical bone components than others.

In vitro bone marrow cultures. Red marrow was obtained from the long bones (tibia, humerus, femur) of an adult New Zealand female rabbit by surgical osteotomy. The samples were pooled and diluted 1:1 with Hank's solution. Aliquots of 0.5 mg (2 × 10^6 cells) were incubated in 5 ml Chromosome 1A Culture Medium (Gibco) with PHA at 37° under an at-

TABLE 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) $P < 0.02$	21.9 ± 5.9 (5) 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.

mosphere of 5% CO_2, 95% O_2. The cultures were harvested at 24, 72 and 96 hr, washed with saline and centrifuged (2×), and DNA was precipitated with 1.0 ml 0.6 *N* TCA. The samples were centrifuged at 4000 rpm for 10 min. The supernate was discarded and 0.1 *N* alcoholic $KC_2H_3O_2$ was added to the pellet. Following centrifugation, 1.0 ml 95% ethanol was added to the tubes and they were heated at 60° for 15 min and recentrifuged; the pellets were each placed in 100% absolute ethanol, dried overnight, and dissolved in 500λ NH_4OH. The total DNA of each tube was estimated fluorometrically by the method of Kissane and Robins (13).

Results and Discussion. Table I shows that in both the inbred and outbred rabbit strains, the autografts had equal osteoinductive potential. New fiber bone comprised 25–35% of the surface area of the implants (Fig. 1). The marrow-free washed implants of allogeneic bone and implants of allogeneic bone with dead marrow cells (frozen–thawed 3×) were completely noninductive; these grafts were being resorbed by large multinucleated osteoclasts at the end of 5 wk.

The Table also shows that composite grafts of ~40 mg wet wt allogeneic bone require at least 50,000 autologous marrow cells in order to achieve an inductive response equivalent to the autografts. Only about 10% of the graft surfaces were covered with new bone when the composite grafts (30 mg allogeneic bone) held 10,000 cultured (96 hr) marrow cells. The ability of the culture system to maintain marrow cell viability for 96 hr was probably satisfactory since the DNA content of the PHA-stimulated cultures increased by a factor of four within 96 hr (Fig. 2).

The *in vivo* PHA experiment permitted an additional insight into the mechanism of the osteoinductive process, for it suggested that composite graft success was indeed dependent upon having a critical number of osteoprogenitor cells on the bone allograft surfaces. The performance of the autografts and of the composite grafts with 50,000 marrow cells was not improved by injections of PHA, but the osteoinductive capacity of the composite grafts with 10,000 marrow cells was normalized. Approximately 30% of the bone surfaces showed new bone formation after *in vivo* PHA stimulation. In these experiments, the action of PHA was most probably mitogenic rather than immunosuppressive. Pretreatment with PHA prior to antigenic stimulation may result in immune suppression of humoral antibody formation (14–17, 20), but it has little capacity to confer immunity when administered at or after the time of challenge (14, 15, 18–20). This study by its nature could not approach problems of the antigenicity of the PHA *per se* (21) and the question of whether the lectin increased the production of blocking antibodies against the weak transplantation antigens of al-

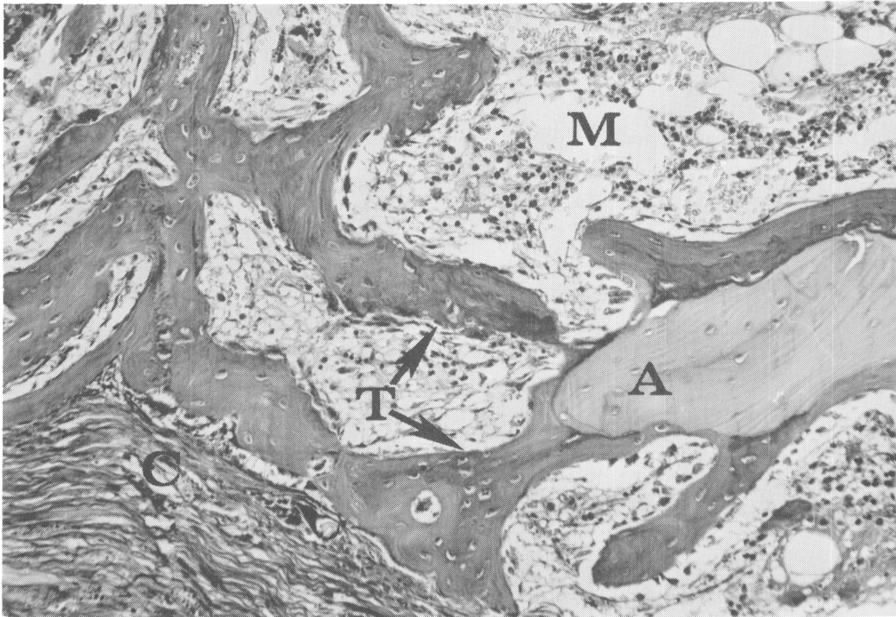

FIG. 1. Photomicrograph of an area of osteoinduction in a heterotopic 5-wk composite graft of allogeneic bone—autologous marrow. A = allogeneic bone trabeculum; T = newly formed trabeculae or fiber bone; C = connective tissue of the host bed; M = marrow. Hematoxylin, Eosin, Azure II stain (250×).

logeneic collagen in the composite grafts so that they had a heightened inductive action.

In a previous publication dealing with the fate of composite grafts (7), we used tritiated thymidine (^{3}HTdr) as a permanent cell marker, and the results suggested that the targets of the inductive stimulus provided by allogeneic bone could have been the reticuloendothelial cells of the autologous marrow as well as the undifferentiated osteogenic precursor cells (fibroblasts?) in the host bed. There is additional convincing evidence from ^{3}HTdr parabiotic studies with rats (22) and other model systems (23) that osteogenic precursor cells are derived from perivascular connective tissue cells. The original studies on composite grafts by Burwell (5, 6) and others (7–9) also offered no clear cut proof that the autologous reticuloendothelial cells in the marrow were the ones induced to become osteogenic—although this interpretation was implicit in the failure of implants of washed xenogeneic (10) or allogeneic bone (24) without marrow to induce new bone. In the present study the implants of allogeneic

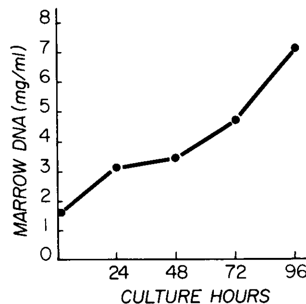

FIG. 2. A plot of the increase in the DNA content of PHA-stimulated *in vitro* cultures of rabbit marrow *vs* time.

bone infiltrated with killed marrow were also noninductive. Moreover, preliminary attempts in this laboratory to use host Strain III rabbits injected with ^{3}HTdr 2X/wk for 2 wk, in order to label the potentially osteogenic cells in the host bed before transplantation, failed to demonstrate that any of the osteocytes in the new bone in composite grafts were derived from those cells.

The studies reported in this paper, therefore, provide additional new evidence that the osteoinductive cells in composite grafts

are mobilized from the marrow elements, and that the target cells of the presumed BMP are the undifferentiated reticuloendothelial or perivascular connective tissue elements. The ability of allogeneic bone to stimulate osteoinduction appears to depend upon having a critical number of cells in the immediate vicinity of the bone substrate, and the presumptive bone inductive protein may operate in part to promote new bone formation in composite grafts by initially stimulating cell division in the target marrow cells.

Summary. The osteoinductive capacities of bone-marrow autografts, washed marrow-free bone allografts, and composite grafts of washed allogeneic bone impregnated with viable or killed autologous marrow cells have been compared in a heterotopic site in rabbits. Composite grafts ($\sim$40 mg allogeneic bone) with viable marrow performed equally well as autografts, providing that these implants contained at least 50,000 marrow cells. Composite grafts with dead marrow were noninductive, and grafts with 10,000 viable marrow cells produced much less new bone than grafts with 50,000 cells. Postoperative treatment of host rabbits with PHA-P increased the osteoinductive capacity of composite grafts with initially subcritical numbers of marrow cells to control levels. These results indicate that the osteoinductive cells in composite grafts are mobilized from the marrow elements rather than from the undifferentiated cells in the host bed.

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