Preliminary Observations on Bone Isografts within Diffusion Chambers.* (23938)

PAUL GOLDHABER† (Introduced by Roy O. Greep)
Department of Oral Pathology, Harvard School of Dental Medicine, Boston, Mass.

A recent historical review(1) of autogenous and homogenous bone grafts indicates real uncertainty as to the origin of newly formed bone and cartilage around the graft. Most investigators favor the concept that new bone arises from surrounding host connective tissue rather than from surviving cells of the graft. Some proponents of this "metaplasia theory" believe that *all* elements of a bone transplant die and are replaced by host tissue —even in *autogenous* bone transplants(2). Indirect evidence, however, supports the hypothesis that new bone is formed by living cells of the graft. Ham and Gordon(3) transplanted bone chips into muscle following a multiple freezing and thawing procedure designed to kill bone cells. No new bone was found adjacent to these chips as compared with untreated bone chips, indicating that new bone was derived from surviving osteogenic cells rather than from host connective tissue. Experiments in our laboratory utilizing fresh and autoclaved young mouse calvaria implanted subcutaneously into 6-week-old mice have yielded similar results (unpublished data). Although both experiments confirm the necessity for utilizing fresh, untreated bone to obtain new bone formation in these sites, neither experiment provides direct proof as to origin of new bone-forming cells. It is possible that *living* cells of the implant "induce" host cells to become osteogenic. In view of difficulties inherent in interpretation of histological evidence presented heretofore, we have attempted a different approach to this problem, whereby it is possible to "partition off" living implants within "diffusion chambers"(4) which allow diffusion of nutrients and metabolites through a porous filter between host tissue and implant *without the passage of cells*. Under these conditions, new bone formation could conceivably occur in the following locations: 1) *Inside chamber exclusively,* indicating that surviving cells of implant are capable of osteogenesis, whereas metaplasia of host connective tissue does not occur. 2) *Outside chamber exclusively,* indicating that although the implant does not retain the capacity to form bone, it is capable of exerting an effect across the filter on surrounding host connective tissue, resulting in differentiation of bone-forming cells. 3) *Inside and outside the chamber,* indicating that newly formed bone occurs as result of osteogenesis of cells of implant plus those of host.

Material and methods. Calvaria of 2 to 3-day-old Swiss albino mice of inbred Webster strain were removed aseptically, placed in Petri dish containing 100 units each of penicillin and streptomycin/ml of Gey's Balanced Salt Solution and divided in half along the median suture. Each half was transferred to a diffusion chamber which had been partially prepared by gluing a Millipore Filter‡ to one side of a thin plastic ring. After insertion of the fragment into the open chamber, it was sealed by attaching a second filter to the open side of the ring. To minimize chamber breakage (which occurred frequently during initial experiments) 2 modifications were made: 1) plastic rings, 1.5 mm thick and center hole 12.5 mm in diameter, were made with a wide flange (4.5 mm) to allow greater surface area for gluing, and 2) stronger, nylon-reenforced Millipore Microweb Filters (19 mm in diameter, 150 μ thick and 0.45 μ pore size) were utilized. Six-week-old mice of same strain as donor tissue were utilized as hosts for chamber implants. Following anesthetization with sodium pentobarbital, an incision was made along right dorsal side of each animal. The skin was separated from underlying muscle with blunt dissection and a pouch created for

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† U.S.P.H.S., Senior Research Fellow.

‡ Obtained from Millipore Filter Corp., Watertown, Mass.

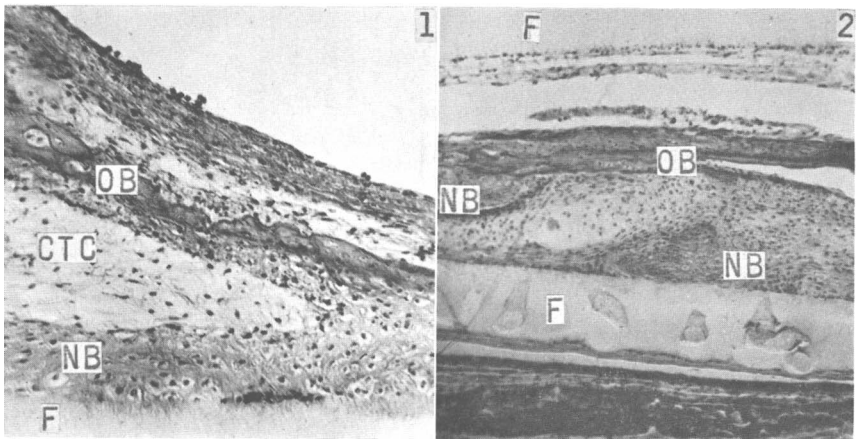

FIG. 1 (× 100). 4-wk chamber implant showing original curved bone of calvarium within chamber (one filter removed). Note loose connective tissue cells which migrated from implant and osteo-chondroidal tissue on inner surface of filter. Symbols: F = filter, OB = original bone, NB = new bone, OC = osteo-chondroid, CTC = connective tissue cells.

FIG. 2 (× 60). 8-wk chamber-implant with both filters in place. The thin original bone is covered in several areas by new bone. In addition, new bone has been laid down on inner surface of filter. "Holes" within lower filter are cross-sections of nylon reenforcement.

reception of diffusion chamber. After insertion of chamber, the incision was closed with nickel-silver wound clips. The animals were sacrificed at intervals of 2, 4 or 8 weeks following implantation, then the chambers and adherent host tissues were removed and fixed overnight in 10% neutral formalin. Following fixation, the plastic rings were cut away prior to decalcification with formic acid and sodium citrate(5). During handling procedures leading to paraffin blocking, any filter not adherent to the tissue within the chamber would tend to separate from the specimen and pull away. However, breakage of the chamber *in vivo* was readily detectable histologically, usually as infiltrating host blood vessels. The following preliminary results are based on histological examination of hematoxylin and eosin stained slides of 20 successful chamber implants.

Results. Marked growth and proliferation of cells from the original transplant occurred within the chambers. Differentiation of cells into bone or bone-like tissue occurred as early as 2 weeks after implantation, and exclusively *within* all chambers on inner surface of filter, surface of original bone, and, occasionally, as scattered islands within the loose connective tissue.

Fig. 1 represents a 4-week implant showing original dark-staining curved bone of calvarium within a chamber (one filter removed). Loose connective tissue cells have proliferated from implant, bridging the gap between original tissue and adherent filter. Cell differentiation has occurred on inner surface of filter, giving rise to new, bone-like tissue containing cartilage elements. Although cell migration through the filter was never noted, partial penetration of cytoplasmic extensions occurred frequently. In some areas a distinct, thin basophilic zone, (possibly an acid mucopolysaccharide) could be distinguished within the filter immediately adjacent to newly formed bone-like tissue.

Further organization of loose connective tissue was found in older, 8-week chambers, Fig. 2, (both filters in place). In several areas, the thin, original bone appears covered by new bone. In addition, new bone with occasional cartilage-cells may be seen on inner surface of filter.

Higher magnification of this area (Fig. 3) shows osteo-chondroidal type of tissue laid down on filter of a 4-week chamber implant. Old bone may be recognized readily in upper portion of photomicrograph by its dark-staining appearance and relatively poor cellularity. Fig. 4 shows the more compact, bone-like tissue found on filter of an 8-week chamber implant. Large cartilage-like cells are present within this tissue as well.

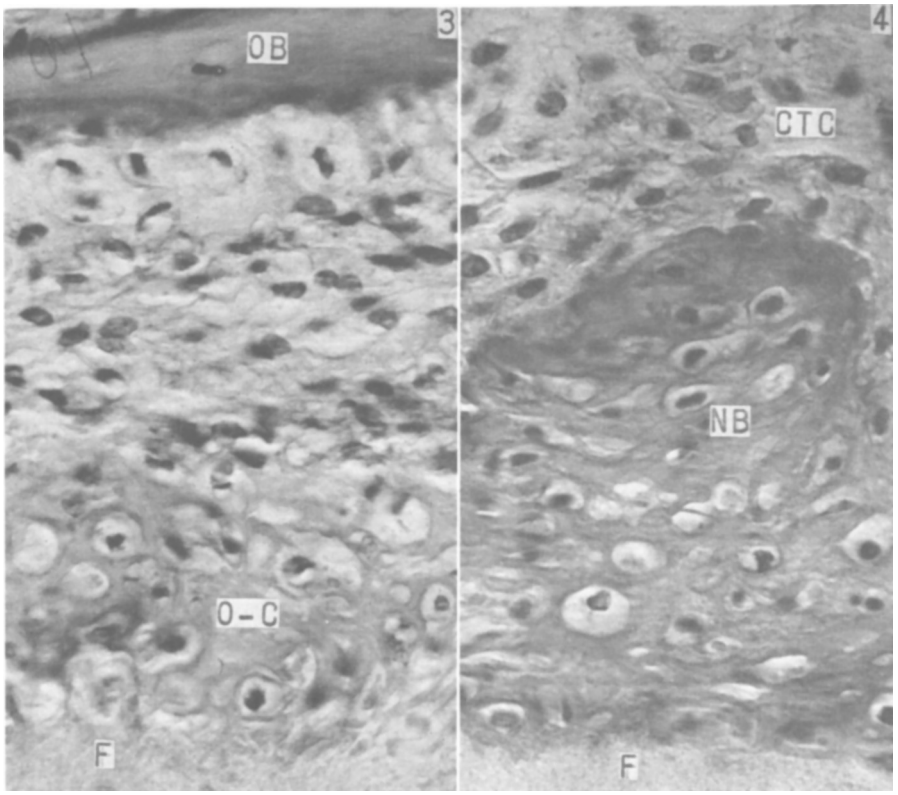

FIG. 3 (× 430). Higher magnification of Fig. 1. Note osteo-chondroidal tissue laid down on inner surface of filter.
FIG. 4 (× 430). Higher magnification of new bone on filter.

Newly differentiated tissue formed in relation to the original bone appeared somewhat more typical of young, active bone. Fig. 5 shows a 4-week chamber with new bone formed around cut edge of old, dead bone. Occasionally, such apposition of new bone filled the entire space between old bone and filter, as found in the 8-week chamber shown in Fig. 6.

Discussion. Our findings provide additional evidence that some cells of bone isografts not only survive, but show excellent ability to proliferate and form new bone and cartilage. Although failure to find any new bone or cartilage on the host side of intact filters may suggest that the "metaplasia theory" is untenable, the possibility cannot be excluded that induction may be responsible for some of the new bone found surrounding a fresh bone autograft in *direct physical contact* with host connective tissue. It should be noted that separation of embryonic inductor and reacting tissue by thin artificial barriers has been shown to block induction *in vivo*. However, diffusible inductor substances can be clearly demonstrated *in vitro*(6). Grobstein(7), working with thin filters *in vitro* has shown that induction with various types of tissue can occur despite a cytoplasmic separation of 20 μ. He suggests that induction may involve interaction between intercellular materials, such as proteins and complex polysaccharides which pass through the filter(8). In view of his findings, it is possible that maintaining chambers in animals for longer periods or using thinner filters might result in formation of bone on the outside of chambers through a mechanism involving bone induction.

It is of interest that "inductive" properties have been ascribed to bone homografts to explain their succesful application in bone-healing studies. It has been suggested that bone

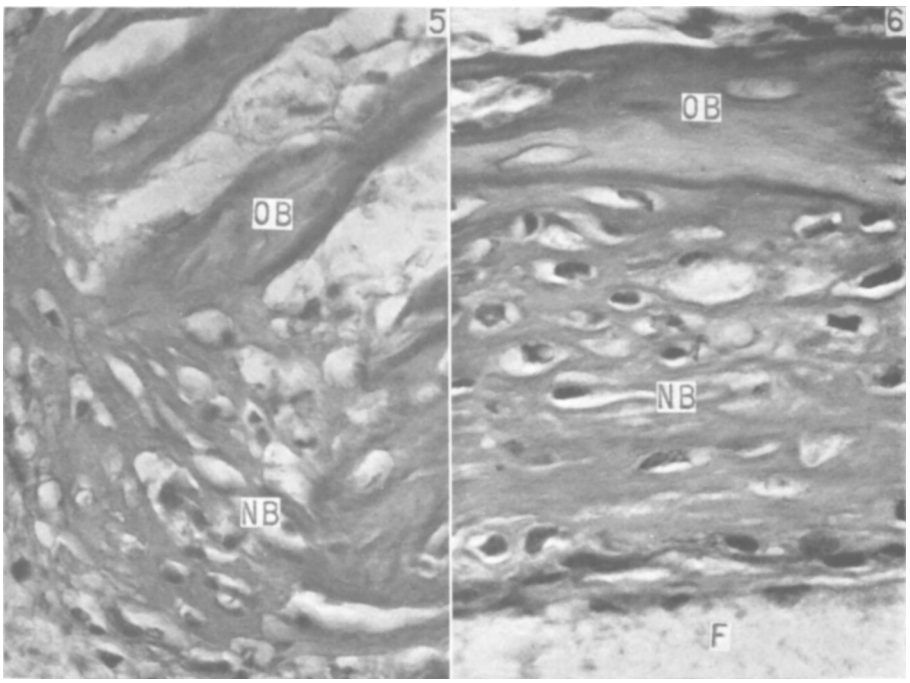

FIG. 5 (× 430). 4-wk chamber-implant with new bone formed around cut edge of old, dead bone.

FIG. 6 (× 430). 8-wk chamber-implant with apposition of new bone on surface of old bone. Note cytoplasmic extensions penetrating into filter.

homograft "provides a stabilizing scaffolding for ingrowing, undifferentiated, mesenchymal cells which, in turn, may be *induced* to become osteogenic by the physiochemical status of the very lattice upon which they grow(9)." Similarly, one might argue that the cellulose ester filters of our chambers have "inductive" properties in view of the new bone and cartilage laid down on their inner surfaces. However, failure of filter to induce host connective tissue to lay down bone and cartilage on the outside of the filters seems to preclude such a mechanism. In contrast to host connective tissue cells, the fibroblast-like cells migrating from original bone implant within the chamber have retained the ability to form bone and cartilage. These reversible changes in cell morphology and differentiation suggest that our findings might be regarded as examples of *cell modulation*(10) rather than induction.

Summary. 1. Surviving cells of bone isografts implanted subcutaneously within diffusion chambers form new bone and cartilage on the surface of original implant and on inner surface of filter. 2. No new bone formation was observed on exterior of filter. 3. It is suggested that the results obtained within diffusion chambers are due to cell modulation rather than induction.

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