Acta Orthop. Scand. *58*, 54–60, 1987

Incorporation of nonviable bone grafts

Autoclaved autogeneic and frozen allogeneic bone grafts compared in the rabbit

In 14 adult rabbits the middle third of the ulna was resected bilaterally followed by reimplantation of resected bone after autoclaving on one side and transplantation of allogeneic bone on the other. In 7 animals the bilateral implants were supplemented with allogeneic bone matrix. The reconstructions were studied in vivo by serial radiography, scintigraphy, and bone mineral determination. The animals were killed at 16 weeks, and the ulnar reconstructions further studied by high resolution radiography, ^{45}Ca autoradiography, and histology. In both types of nonsupplemented reconstructions, new bone formation was poor; nonunion occurred in three out of seven autoclaved reimplants and in five out of seven allogeneic transplants. Supplemented with allogeneic bone matrix, both types of reconstructions exhibited abundant new bone formation and complete incorporation of all implants.

Enhancement of new bone formation is probably more important than the type of nonviable bone graft chosen for reconstruction of large skeletal defects.

Peter Köhler
Jan-Erik Glas[1]
Stig Larsson[2]
Andris Kreicbergs

Department of Orthopedics, Karolinska Hospital, [1]Department of Pathology and Cytology, Sabbatsberg Hospital, [2]Department of Hospital Physics, Karolinska Hospital, S-104 01 Stockholm, Sweden

In recent years, local resection has become increasingly applied in the treatment of malignant bone tumors as an alternative to ablative surgery (Chao & Ivins 1983, Enneking et al. 1980, Koskinen et al. 1979, Ottolenghi 1972, Nilsonne 1969, Parrish 1966). However, the mandatory requirement for radicality often leads to extensive resections causing considerable problems of reconstruction. Allogeneic bone grafting of large skeletal defects is elaborate and expensive involving a complicated system for obtaining cadaveric bone, for specimen storing and matching between donor and recipient (Mankin et al. 1982, Friedlaender 1982).

Occasional reports indicate that reimplantation of tumorous bone after autoclaving may afford a combined means for tumor devitalization and skeletal reconstruction (Smith & Simon 1975, Johnston et al. 1983, Thompson & Staggall 1956). The procedure, by its simplicity, seems to offer several advantages to allogeneic bone grafting. In a previous study in the rabbit (Köhler & kreicbergs 1987) we found that autoclaved autogeneic bone grafts when supplemented with allogeneic bone matrix, known to be a potent inductor of new bone formation (Oikarinen & Korhonen 1979, Urist 1967, 1971, 1974, Wittbjer 1983), consistently incorporated as opposed to those nonsupplemented. However, it remains unclear whether autoclaved autogeneic bone differs from frozen allogeneic bone with respect to incorporation. We have now compared autoclaved autogeneic and frozen allogeneic bone grafts, supplemented with allogeneic bone matrix and nonsupplemented, in the reconstruction of large skeletal defects.

Materials and methods

Twenty-three adult male rabbits (New Zealand White) were anesthetized by i.m. injection containing a mixture of fluanizonium 10 mg and fentanyl citrate 0.315 mg/mg (Hypnorm, Leo), given in a dose of 0.3 ml/kg body weight combined with diazepam 1.5 mg/kg (Valium, Roche). The lower forelegs were depilated and surgery was carried out under sterile conditions. Templet-guided transverse resection of the middle third (20 mm) of ulna including the periosteum was performed bilaterally.

The ulnar defects were reconstructed by the resected specimen after autoclaving (121°C/20 min) on one side and by an allogeneic ulnar graft on the other side. The allogeneic grafts were collected from female rabbits, raised by a different breeder, and subsequently stored in a deep freeze (−70°C) under sterile

conditions before implantation. The periosteum was removed from all the grafts.

In 13 of the 23 animals, the bilateral reconstructions were supplemented with 175 mg allogeneic bone matrix *both* as granulate (approx. 25 mg) in the osteotomies and as pieces (approx. 150 mg) along the reconstructions.

Allogeneic bone matrix (ABM) was prepared from cortical diaphyseal bone of fresh rabbit cadavers after removal of the metaphyses, marrow, and soft tissues. The bones were cut into pieces, which were decalcified in 0.6M HCl for 24 h, rinsed 3 times in water, defatted in chloroform-methanol (1:1) for 1 h, rinsed 3 times in methanol and additionally 3 times in water. The entire process was performed at +4°C. The prepared pieces of allogeneic bone matrix were lyophilized to a constant weight. Some of the pieces were milled to 1 mm fragments in a Spex Freezer Mill in liquid nitrogen. The preparations were disinfected by dry ethylene oxide exposure for 24 h and subsequently ventilated in dry air for 1 week to remove any remaining gas.

No internal fixation was used, and postoperatively, the animals, which were kept in cages, were allowed full weight bearing.

Incorporation of the implants was studied in vivo (in Hypnorm-Valium anesthesia) 2, 4, 8, 12, and 16 weeks postoperatively by a) radiography, b) scintigraphy, c) bone mineral density determination (except at 2 weeks). At 16 weeks the animals were killed by an overdose of i.v. pentobarbital sodium (60 mg/ml), and the ulnar specimens were collected for postmortem investigation that included d) high-resolution radiography, e) autoradiography, and f) histomorphology.

Radiography. The forelegs were placed in plastic tubes permitting reproducible projections. At examination the position of each implant in relation to the olecranon was determined in mm with a plane parallel ruler placed along the foreleg. Hence, the region of interest for subsequent bone mineral determination (see below) could be accurately defined in each animal.

Scintigraphy. The animals were given 50 MBq ^{99m}Tc-MDP i.v. 2 h before the investigation, which was performed (forelegs in plastic tubes) with a maxicamera 400T (General Electric) connected to a Gamma-II image-processing system (Digital Equipment). A total of 300,000 counts per 64 x 64 pixel matrix frame (pixel size = 6.3 x 6.3 mm) was acquired to obtain approximately 10,000 counts in the chosen regions of interest. The uptake of ^{99m}Tc-MDP was determined for an 120 pixel rectangular region of interest over each lower foreleg. In addition, the number of counts in a 16 pixel rectangular area over each shoulder was assessed as a reference. Thus, the mean uptake of both shoulders was calculated, and the ratio between the uptake of each foreleg and the reference was determined each time for each animal.

Bone mineral density determination. The measurements were performed in a Bone Density Scanner (Nuclear Data 1100), which permits quantitation of the total amount of calcium hydroxy apatite in the scanned part of the specimen. The method is based on transmission measurements using ^{125}I as the emission source. The calculated absorption provides a measure of the bone mineral content (Sorenson & Cameron 1976). The animals were placed in a specially designed box for measurement of the forelegs in water to compensate for differences in soft-tissue absorption. The position of each implant was deduced from radiographs taken with a ruler indicating the distance between the olecranon (easily palpated) and each of the two osteotomies. Two 5 mm wide, depilated implant areas were scanned perpendicular to the longitudinal axis of each lower foreleg. The mean value of the two determinations was calculated as a measure of the total bone mineral content of each implant area.

High-resolution radiography. Radiographs providing very detailed depictions of postmortem specimens were obtained by using high-resolution film (Kodak Industrex M) in a Hewlett Packard Faxitron at 35kV/144 mAs.

Autoradiography. One animal in the ABM and in the nonsupplemented group was given 30 MBq ^{45}CaCl i.v. 2 days before death. The ulnar specimens were collected for embedding in carboxy methylene cellulose and sectioning (35 μ) in a cryomicrotome. The sections were mounted on tape and firmly attached to film (Agfa Gaevert Structurix D7) for 7 days. The films were developed and subsequently analyzed with respect to distribution of ^{45}Ca incorporated in the specimens (Rohlin et al. 1977).

Histomorphology. After fixation (10 per cent buffered neutral formalin) and decalcification (mixture of 0.5 M formic acid and 1.48 M HCl), the ulnar specimens were grossly cut, subsequently paraffin embedded, sectioned at 10 μ, and stained in Weigart's hematoxyline and van Gieson for microscopic analysis.

Calculations and statistics. Numerical data from scintigraphy and bone mineral determinations were used a) to assess changes in relation to time in each type of implant (thus, the means of the recorded values for each type of implant was calculated at 2, 4, 8, 12, and 16 weeks postoperatively) and b) to compare autoclaved reimplants and allogeneic transplants. Thus, the ratio between the two values *in each animal* at different times was calculated, and the means of the ratios analyzed by paired two-tailed Student's *t* test. *P* values >0.05 were considered not significant.

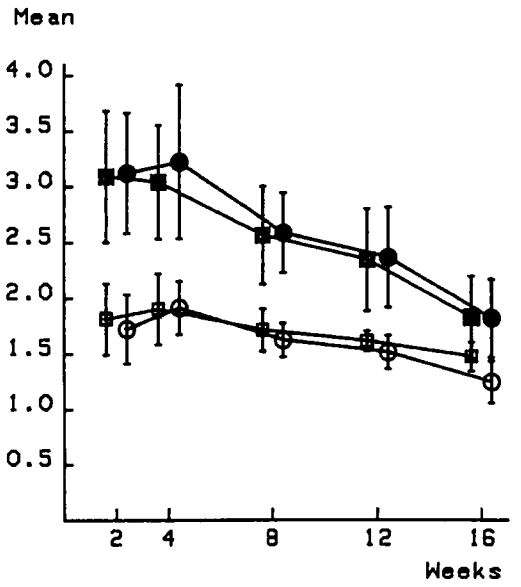

Figure 1. ^{99}mTc-MDP uptake in relation to time. Mean uptake of autoclaved reimplants (squares) and allogeneic transplants (circles). Filled symbols denote ABM – supplementation.

Two animals died within 48 hours postoperatively showing signs of pulmonary hypoxia. One animal died during anesthesia 4 weeks postoperatively. Another 6 animals had to be excluded because of unilateral fracture of the radius. Thus, a total of 14 animals, i.e., 7 with nonsupplemented reconstructions and 7 with reconstructions supplemented with allogeneic bone matrix (ABM) completed the entire study.

Results

Serial radiography essentially showed the same features in both types of nonsupplemented reconstructions, i.e., some new bone formation around the osteotomies, but almost none along the implants. There were signs of nonunion (at least in one osteotomy) in three reconstructions with autoclaved reimplants and in five with allogeneic transplants. In the reconstructions supplemented with ABM, there were clear signs of new bone formation in the osteotomy areas already at 2 weeks, scattered new bone covering the entire length of the implants at 4 weeks, with subsequent consolidation between 8 and 16 weeks. At the end of the study, the allogeneic implants appeared less well outlined than the autoclaved ones. Nonunion did not occur in any of the 14 ABM-supplemented reconstructions.

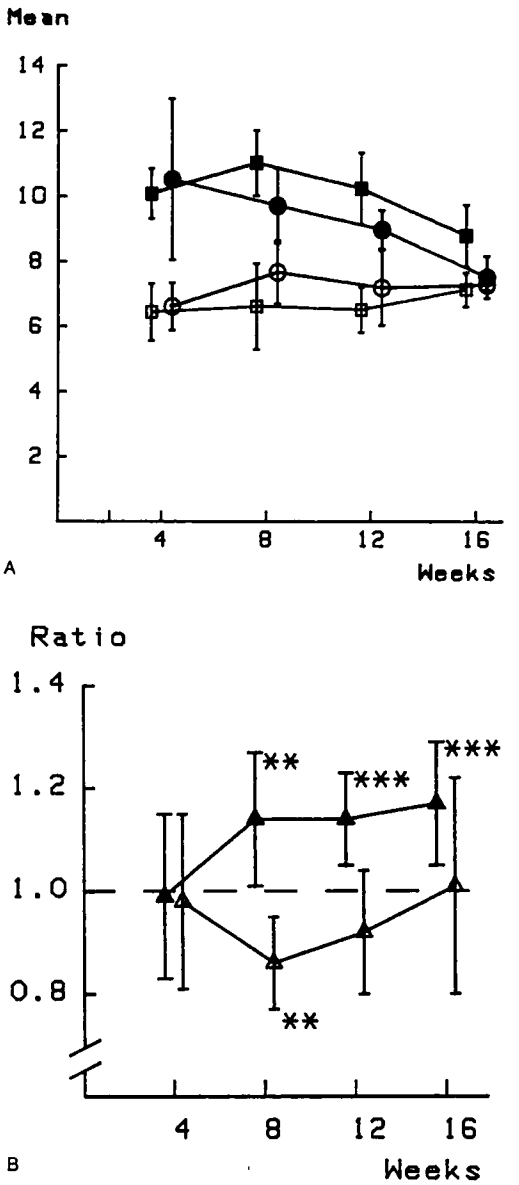

Figure 2. Bone mineral content in relation to time. A. Mean content of autoclaved reimplants (squares) and allogeneic transplants (circles). B. Content ratio (triangles): autoclaved reimplants/allogeneic transplants. Filled symbols denote ABM – supplementation.

Serial scintigraphy of the compared nonsupplemented reconstructions showed no differences. There was increased uptake 2 and 4 weeks postoperatively, mainly over the osteotomies, subsequently decreasing to almost the base level. In both types of ABM-supplemented reconstructions, the uptake was increased over the entire implant area 2, 4, as well as 8 weeks

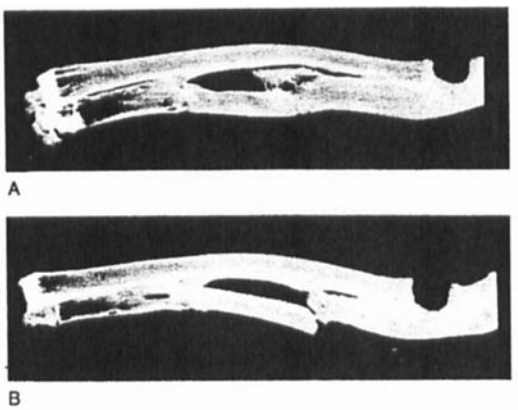

Figure 3. High-resolution radiography of nonsupplemented reconstructions with autoclaved reimplants and allogeneic transplants showing A incorporation, and B nonunion.

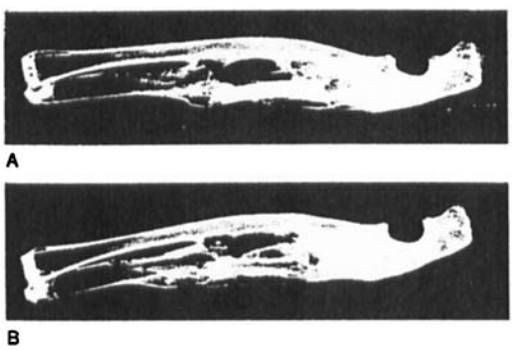

Figure 4. High-resolution radiography of ABM-supplemented reconstructions: A autoclaved reimplant, B allogeneic transplant.

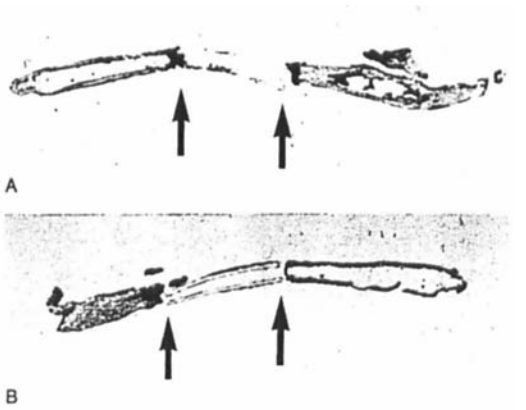

Figure 5. Autoradiographs showing amount and distribution of ^{45}Ca deposition in nonsupplemented reconstructions: A autoclaved reimplant, B allogeneic transplant. Implant area between arrows.

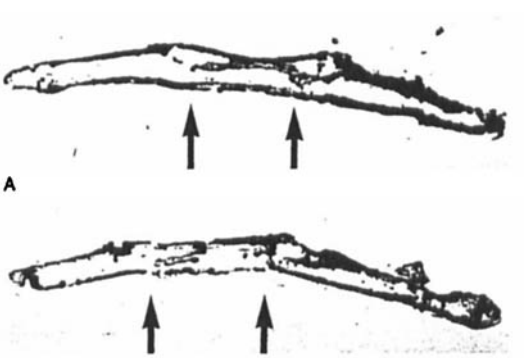

Figure 6. Autoradiographs showing amount and distribution of deposition in supplemented reconstructions: A autoclaved reimplant, B allogeneic transplant. Implant area between arrows.

postoperatively, followed by a slow return to almost normal uptake (Figure 1).

Comparison of the two types of nonsupplemented implants showed no difference in bone mineral content except at 8 weeks, when the allogeneic transplants had higher values (Figure 2). In the ABM-supplemented reconstructions the bone mineral content was higher in the autoclaved reimplants than in the allogeneic transplants at 8, 12, as well as 16 weeks, postoperatively.

High-resolution radiography (Figures 3 and 4) of removed specimens essentially confirmed the findings of conventional radiography, but more clearly illustrated the amount and distribution of new bone in the specimens. Further, it convincingly showed that there was nonunion in 8 of the 14 nonsupplemented reconstructions, but in none of the ABM-supplemented ones.

Autoradiography showed moderate deposition of ^{45}Ca in the osteotomy areas of the nonsupplemented reconstructions, but only a thin layer along both types of implants (Figure 5). In both types of ABM-supplemented reconstructions there was abundant ^{45}Ca deposition in the osteotomy areas and also along the implants corresponding to newly formed bone. There appeared to be some deposition in the implants themselves. No difference in amount and distribution of ^{45}Ca could be demonstrated between the two types of ABM-supplemented reconstructions.

Histologic analysis disclosed no major differ-

Figure 7. Nonsupplemented autoclaved reimplant with thin layer of appositional new bone.

Figure 9. ABM-supplemented allogeneic transplant. High power magnification of junctional area (arrows) between new appositional bone (N) and implant bone (I).

ences between the nonsupplemented reconstructions. Both the autoclaved reimplants and the allogeneic transplants appeared nonviable, for no cell nuclei in the lacunae or any distinct vessel structures could be demonstrated. Another common feature was resorption, which appeared somewhat more pronounced in the allogeneic transplants. Along both types of implants there was a thin, irregular, appositional layer of presumably new viable bone in intimate contact with implant bone (Figure 7).

Histologic analysis of the ABM-supplemented reconstructions showed abundant new bone around the implant areas. A thick layer of appositional new bone in intimate contact with implant bone (Figure 8) was consistently found in both types of reconstructions. In the junctional area, particularly in the allogeneic reconstructions, new bone appeared to have replaced implant bone (Figure 9). Thus, the only difference observed between the compared implants was the degree of resorption, which appeared somewhat more pronounced in the allogeneic transplants. The remainders of the implants appeared to be nonviable in both types of reconstructions.

All the specimens, regardless of whether ABM-supplemented or not, were surrounded by a highly vascular fibrous tissue of nondegenerative type with a few areas of fatty necrosis, but no signs of inflammatory reaction.

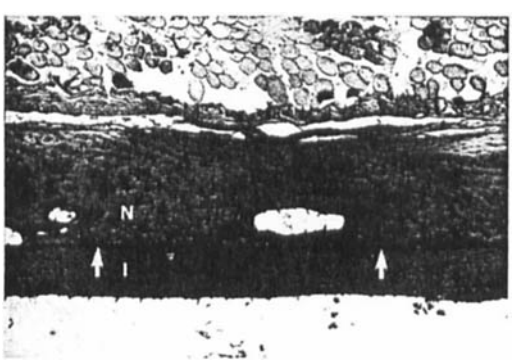

Figure 8. ABM-supplemented autoclaved reimplant showing thickness of new appositional bone (N) in relation to cortex of implant bone (I). Junction denoted by arrows.

Discussion

Our study indicates that there is no decisive difference between autoclaved autogeneic and frozen allogeneic bone when used for reconstruction of large skeletal defects; both types of implants were found to be associated with poor new bone formation and a high rate of nonunion. However, when supplemented with allogeneic bone matrix, the reconstructions exhibited abundant new bone formation, and both types of implants incorporated consistently. Our findings indicate that enhancement of new bone formation probably is more important than the type of nonviable bone graft chosen for reconstruction.

The only variable showing a difference between autoclaved reimplants and allogeneic transplants proved to be bone mineral content; in the nonsupplemented implants the bone mineral content was higher in the allogeneic

grafts at 8 weeks postoperatively. This might reflect an increase in bone mineral content of the allogeneic transplants and/or a decrease in the autoclaved reimplants. Whether the observed difference solely at 8 weeks should be attributed to true changes in the bone mineral content of the implants or merely represents a coincidental finding, in spite of statistical significance, remains unclear. In the ABM-supplemented implants, there was a consistently higher bone mineral content in the autoclaved reimplants compared with the allogeneic transplants from 8 weeks and onwards. Noteworthy, both types of implants showed a slow decrease in bone mineral content during this period. This indicates that resorption occurred in both types of ABM-supplemented implants, although at different rates. The reason for the observed difference in resorption remains obscure, but may be attributed to differences in antigenicity of the compared implants and/or physical effect of previous freezing and autoclaving.

An important factor for the development of the several nonunions in the nonsupplemented reconstructions, apart from low osteogenic activity, applies to the surgical technique. In a previous study (Albrektson 1971) on internally fixed allografts after subperiosteal resection of ulnar segments in the rabbit, the rate of nonunion was only 10 per cent. Thus, it appears that nonviable implants incorporate better if recipient periosteum is preserved and the grafts are properly stabilized. However, local resection of bone tumors should include the periosteum. In our study it was, therefore, removed, which entails a significant loss of osteogenic tissue (Kirkup 1965). In spite of this fact and deficient fixation, all ABM-supplemented implants incorporated.

There are reasons to believe that the combination of two grafts, i.e., a nonviable bone specimen and a bone inductive substance, is superior to either of the two for reconstructions of skeletal defects. Merely substitution with allogeneic or autogeneic bone matrix without a bone implant has been reported to be associated with incomplete osseous bridging in 19 and 25 per cent, respectively (Tuli et al. 1978, Wittbjer 1983). However, other experiments applying an analogous principle to the one now presented, i.e., a combination of a bone inductive substance and a skeletal substitute using surface decalcified allogeneic bone, have given similar good results (Dubuc & Urist 1967, Gupta & Tuli 1982, Urist et al. 1975, 1981). Nonviable bone implants may be assumed to play an important role as temporary stabilizers and possibly also as primers for new bone formation.

We conclude that autoclaved autogeneic and frozen allogeneic bone are essentially equivalent as grafts. Addition of allogeneic bone matrix seems to improve the incorporation of such grafts. For reconstruction of large skeletal defects, autoclaved autogeneic bone is simpler to use and therefore appears preferable to allogeneic bone.

Acknowledgements

This study was supported by grants form the Swedish Cancer Society (Grant No. 84:96) and the Jonas Björnberg Memorial Fund.

References

Albrektson, B. (1971) Repair of diaphyseal defects. Experimental studies on the role of bone grafts in reconstruction of circumferential defects in long bones. Thesis. University of Gothenburg, Sweden.

Chao, C. Y. & Ivins, J. C. (1983) *Tumor prosthesis for bone and joint reconstruction.* Georg Thieme Verlag, Stuttgart, New York.

Enneking, W. F., Eady, J. L. & Burchardt, H. (1980) Autogenous cortical bone grafts in the reconstruction of segmental skeletal defects. *J. Bone Joint Surg.* **62-A,** 1039–1058.

Friedlaender, G. E. (1982) Bone-banking. *J. Bone Joint Surg.* **64-A,** 307–311.

Dubuc, F. L. & Urist, M. R. (1967) The accessibility of the bone induction principle in surface-decalcified bone implants. *Clin. Orthop.* **55,** 217–223.

Gupta, D. & Tuli, S. M. (1982) Osteoinductivity of partially decalcified alloimplants in healing of large osteoperiosteal defects. *Acta Orthop. Scand.* **53,** 857–865.

Johnston, J. O., Harries, T. J., Alexander, C. E. & Alexander, A. H. (1983) Limb salvage procedure for neoplasms about the knee by spherocentric total knee arthroplasty and autogenous autoclaved bone grafting. *Clin. Orthop.* **181,** 137–145.

Kirkup, J. R. (1965) Traumatic femoral bone loss. *J. Bone Joint Surg.* **47-B,** 106–110.

Koskinen, E. V., Salenius, P., Alho, A. (1979) Allogeneic transplantation in low-grade malignant bone tumours. A new operative technique to avoid amputation. *Acta Orthop. Scand.* **50**, 129–138.

Köhler, P. & Kreicbergs, A. (1986) Incorporation of autoclaved autogeneic bone supplemented with allogeneic bone matrix. *Clin. Orthop.* **218**, 247–258.

Mankin, H. J., Doppelt, S. H., Sullivan, T. R. & Tomford, W. W. (1982) Osteoarticular and intercalary allograft transplantation in the management of malignant tumours of bone. *Cancer* **50**, 613–630.

Nilsonne, U. (1969) Homologous joint-transplantation in man. *Acta Orthop. Scand.* **40**, 429–447.

Oikarinen, J. & Korhonen, L. K. (1979) Repair of bone defects by bone inductive material. *Acta Orthop. Scand.* **50**, 21–26.

Ottolenghi, C. E. (1972) Massive osteo and osteoarticular bone grafts. Technic and results of 62 cases. *Clin. Orthop.* **87**, 156–164.

Parrish, F. F. (1966) Treatment of bone tumours by total excision and replacement with massive autologous and homologous grafts. *J. Bone Joint Surg.* **48-A**, 968–990.

Rohlin, M., Larsson, Å. & Hammarström, L. (1977) Distribution of Tc-99m labelled phosphorous compounds, 45Ca and Sr85 in diphosphonate-treated rats. *Acta Radiol Ther. Phys. Biol.* **16** , 513–524.

Smith, W. S. & Simon, M. A. (1975) Segmental resection for chondrosarcoma. *J. Bone Joint Surg.* **57-A**, 1097–1103.

Sorenson, J. A. & Cameron, J. R. (1967) A reliable in vivo measurement of bone-mineral content. *J. Bone Joint Surg.* **49-A**, 481–497.

Thompson, V. P. & Staggall, C. T. (1956) Chondrosarcoma of the proximal portion of the femur treated by resection and bone replacement. *J. Bone Joint Surg.* **38-A**, 357–367.

Tuli, S. M. & Singh, A. D. (1978) The osteoinductive property of decalcified bone matrix. An experimental study. *J. Bone Joint Surg.* **60-B**, 116–123.

Urist, M. R., Silverman, B. F., Buring, K., Dubuc, F. L. & Rosenberg, J. M. (1967) The bone induction principle. *Clin. Orthop.* **53**, 243–283.

Urist, M. R. & Strates, B. S. (1971) Bone morphogenetic protein. *J. Dent. Res.* **50**, 1392–1406.

Urist, M. R., Earnest, F. IV., Kimball, K. M., Di Julio, T. P. & Iwata, H. (1974) Bone morphogenesis in implants of residues of radioisotope labelled bone matrix. *Calc. Tiss. Res.* **15**, 269–286.

Urist, M. R., Mikulski, A. & Boyd, S. D. (1975) A chemosterilized antigen-extracted autodigested alloimplant for bone banks. *Arch. Surg.* **110**, 416–425.

Urist, M. R. & Dawson, E. (1981) Intertransverse process fusion with the aid of chemosterilized autolyzed antigen-extracted allogeneic (AAA) bone. *Clin. Orthop.* **154**, 97–113.

Urist, M. R., DeLange, R. J. & Finerman, G. A. (1983) Bone cell differentiation and growth factors. *Science* **220**, 680–686.

Wittbjer, J. (1983) Bone matrix and bone formation. An experimental study of demineralized bone reimplanted in bone defects. Thesis. pp. 1-44. University of Lund, Sweden.