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**Fresh and Frozen Allogeneic Iliac Crest Transplants in Primates: A Sequential Histopathologic Study**

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FRESH AND FROZEN ALLOGENEIC ILIAC CREST TRANSPLANTS IN PRIMATES: A SEQUENTIAL HISTOPATHOLOGIC STUDY

by

Robert Colin Poulsom, B.S., D.D.S.

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Oral Biology

May 1975
DEDICATION

To my father-in-law, Howard Charles Abbott, for his encouragement, assistance, and forbearance.

To my wife, Candy, for her love, sacrifice, and patience.
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CHAPTER I

INTRODUCTION

In the course of periodontal disease, the infrabony osseous defect is a common occurrence. A variety of surgical procedures have been employed in an attempt to correct these lesions. These include recontouring of the alveolar process by osteoplasty and/or osteoectomy, curettement of the osseous defect in order to gain a new gingival attachment in addition to some bone regeneration in the defect, and the various grafting procedures.

The treatment of advanced periodontal disease presents a problem in that the osseous defects do not lend themselves to corrective treatment by curettement or osseous recontouring alone due to the size, shape, and extent of the lesions at this late stage. When osseous resection techniques are utilized, the removal of both supportive and non-supportive bone in order to eliminate bony craters and non-physiologic architecture results in a serious weakening of the functional supportive mechanism of the teeth. Sufficient supportive structures of a tooth are vital to its preservation.
In recent years there has been a thrust in periodontics toward the replacement of lost periodontal structures by means of grafting procedures. Many materials have been tried including plaster of Paris, sclera, cartilage, collagen, and bone. The rationale behind the use of these materials in osseous defects is that they will increase the predictability and amount of new attachment apparatus regenerated by the induction of new alveolar bone, periodontal ligament, and cementum formation. This approach to periodontal treatment is a much more positive one in that the addition of supportive structures is more desirable and less destructive than their subtraction via osseous resection.

The use of cancellous bone and marrow from the iliac crest in the treatment of osseous defects in the course of periodontal therapy has already been proposed.\(^1,2,3,4,5\) Both fresh and frozen autogenous iliac transplants have been utilized in the treatment of osseous defects. A histologic evaluation and comparison of the healing phenomena of fresh and frozen iliac autografts has been done recently.\(^6\)

There are, of course, several drawbacks to the use of autogenous iliac crest grafts. There is an additional surgical procedure necessitated in order to obtain the bone for grafting from the hip. Also there is some discomfort to the patient which accompanies this added procedure. A possible solution to these problems would be the establishment of a bone bank from
which allogeneic cancellous bone and marrow could be obtained prior to the surgical procedure. This would eliminate the necessity of an additional procedure on the patient but poses some new problems. Very little research has been done on the utilization of allogeneic bone transplants in periodontics. There is the possibility that the transplants will be rejected because of the lack of histocompatibility. No histologic evaluation has been done to study the healing phenomena of allogeneic iliac transplants in periodontal defects. The purpose of this investigation is to study histologically the sequential healing phenomena of fresh and frozen allogeneic iliac cancellous bone and marrow grafts placed into chronic periodontal osseous defects, and to compare the healing process of these grafts in order to determine the presence or absence of any significant difference which could be attributed to the preservation of the allogeneic bone material.
CHAPTER II

REVIEW OF THE LITERATURE

A. BONE GRAFTS

The first reported use of bone grafts in the reconstruction of the supporting tissues during treatment of periodontal disease appeared in 1923. Hegedus reported six cases in which fresh autogenous periosteal-cortical grafts from the tibia were employed in an attempt to reconstruct the alveolar process in cases of severe horizontal bone loss. Intraoral donor sites were attempted first but "great technical difficulties," and severe postoperative pain due to prolonged tissue manipulation while obtaining sufficient bone discouraged this donor site. Buccal and lingual mucoperiosteal flaps were reflected at the recipient site. The chronic inflammatory tissue was removed and the roots were planed. The alveolar crest at the recipient site was curetted in order to stimulate bleeding. The blood aided in retaining the bone graft. After the graft was placed, the flaps were sutured over the graft. Hegedus placed great emphasis on the importance of strict asepsis to avoid osteomyelitis of the tibia. Decreased tooth mobility, pocket eradication, and
radiographic evidence were attributed to bone-building and the regenerative periosteal capacity and were used as his criteria for clinical success.

Murray\(^8\) in 1931 experimented with the use of bone powder and concluded that the calcium in the graft was necessary for the calcification of newly forming bone and cementum. He also stated that the fibrin clot has an affinity for the calcium in the grafted bone and thus aids in the retention of the bone powder.

Beube and Silvers\(^9\) in 1934 resorted to the use of boiled heterogeneous bone powder because they could foresee practical difficulties associated with the use of live autogenous bone. They placed sheep bone powder into fresh surgically created defects in the maxillae of six dogs while the control sites did not receive grafts. The histologic report states that no new bone formation was seen in the control defect, only a fibrous tissue filling of the defect. The experimental sites were filled with "a hard firm substance" which he called early bone formation. They showed that bone particles accelerate osteogenesis. Beube and Silvers attributed this acceleration to the additional calcium supply of the grafted bone which helps to calcify the granulation tissue during the repair phase.

Beube and Silvers\(^10\) in 1936 reported success in using boiled long bone of a cow (devitalized heterogeneous bone powder) in
treating intraoral osseous defects in humans. They claimed that success was due to the fibrin clot which bound the calcium to the area and acted as a matrix for the bovine bone-powder. This calcium can be used in the new bone formation in the periodontal defect.

McGaw and Harbin, 11 in 1934, explained the advantage of including either periosteum, endosteum, or bone marrow in a bone graft. They stated without anyone of these three rich sources of osteoblasts, the new bone growth will be slow and scanty. They believed that being bone marrow, the richest source of osteoblasts, it would act to stimulate more rapid and complete osteogenesis.

Levander, 12 in 1940, suggested that bone marrow undergoing necrosis after its transplantation liberates an inductive substance which has the ability to stimulate osteogenesis by transforming undifferentiated mesenchymal cells into osteoblasts. He thought that the bone fragments did not play an important role in the formation of new bone other than as a liberator of an inducer substance.

In 1949, Beube 13 again experimented with the use of heterografts utilizing boiled cow bone powder. He concluded that the repair process was the same regardless of the presence or absence of the cow bone. The presence of the cow bone, however, did hasten the repair process by providing a more rapid bone and
cementum deposition. He stated that the bone powder supplemented the calcium concentration available from the circulatory system. According to Beube, the bone powder acts as an irritant causing the release of histamine which in turn causes a vasodilatation. The vasodilatation improves the circulation to the graft site and thus healing occurs more rapidly. His study provided a description of the histological healing sequence of the cow bone heterograft. In addition, Beube conceptualized that the cementoblasts and the osteoblasts may possibly be one in the same cell. He further concluded that both of these cells, the cementoblasts and the osteoblasts, were differentiated forms of the fibroblast. He found that the bone powder begins to resorb on day 7 and is completely gone by day 14.

Linghorne and O'Connell, in 1951, studied the repair of periodontal structures after surgically created defects were packed with autogenous bone chips. They stated that the presence of resorbing calcified tissues appear to be a factor in the differentiation of osteoblasts. The osteogenic effect of the grafts is due less to their cellular content than to the calcified intercellular material. They also claimed, like Beube, that there is little difference between osteoblasts and cementoblasts in the reparative process, both originating from undifferentiated mesenchymal cells. No difference could be detected between the material deposited on the grafts which later became bone and that
which became cementum. They claimed that grafts encouraged osteogenesis by stimulation of the growth of new trabeculae and by acting as new islands of ossification. Cross\textsuperscript{15-19} reported on various cases that he treated with autografts, homografts, and heterografts. Radiographic evidence of bone regeneration was presented. The type of graft used in each situation, the method of approach, the type of defect, and the length of time the case was followed post-operatively was not specified, thus, making evaluation of the various types of grafts impossible. Cross suggested that small chips of bone be used in order to diminish the possibility of the formation of a sequestra. He also advocated the use of prophylactic antibiotics post-operatively.

In 1955, Forsberg\textsuperscript{20} reported on the use of non-vital heterogeneous bone called "os purum." It is an ox bone derivative made by the removal of fat, connective tissue and protein so as to leave only the calcium salts and cementing materials. He noted that out of 11 cases, 3 were unsatisfactory, 7 satisfactory, and 1 excellent. Forsberg stated in this paper that he has switched to fresh autogenous bone from the iliac crest for use in intra-osseous defects.

Schaffer,\textsuperscript{21} in 1956, working with rhesus monkeys, reported the successful use of autografts and homografts of cartilage in surgically created periodontal pockets. In 1958, he reported
70 homogeneous cartilage grafts in 26 patients. New bone was reported in 21 of the 70 cases. A new attachment in a position coronal to the preexisting attachment was found in 60 of the 70 cases. In 1957, Schaffer reported the use of autogenous and homogeneous cementum-dentin implants in extraction sites and in surgically created defects in a dog and a monkey. Histologically, he described bone apposition on the surface of the graft particles. What was significant in this study was his statement that small graft particle size was more advantageous to new bone formation than larger particles.

Mineralized materials acting as a calcium source, were often grafted into periodontal defects in an attempt to stimulate osteogenesis. Stallard and Randall in 1959, Toto et al., in 1961, and Glickman and Patur, in 1962, utilized "anorganic bone" (ethylenediamine extracted bone) and Radentz and Collings, in 1965, utilized plaster of Paris. Both materials demonstrated the capacity to stimulate osteogenesis, but neither material gave consistent results.

Schaffer teamed with Pacher, in 1962, described the unpredictability of the resorption of cartilage grafts. In 1964, these authors used cementum and dentin grafts in surgically created defects. They suspended the graft material in an absorbable gelatin sponge matrix (Gelfoam). The results were not consistent. In fact, Gelfoam alone produced the best results.
In 1959, Yuhtanandana created periodontal pockets surgically in dogs. He attempted to simulate the chronicity of naturally occurring pockets by adding self-cured acrylic, tin foil, or surgical cement to the defect and suturing the flap tight over it. He concluded that osteogenesis and connective tissue reattachment can be stimulated by the presence of autogenous cancellous bone.

In 1959, Schneider, Harder and Thompson grafted homologous costal cartilage chips and autogenous and homogeneous bone chips in surgically created supra-bony defects in dogs. They concluded that the homogeneous bone grafts had a greater stimulatory effect on new attachment than did the homogeneous cartilage grafts.

Krömer in 1960 used merthiolate-preserved homografts. Nine grafts were successful while three grafts were expelled prior to healing. In 1963, he reported using this graft material in 30 pockets in patients. Twenty-three were successful, four partially successful, and three failures. Merthiolate was used believing that it would render the graft resistant to infection due to its bactericidal and fungicidal properties.

The origin of osteoblasts in the bone grafts was a major subject of interest at this time. Three possible sources of osteoblasts have been proposed: Bruns suggested they may arise from pre-existing osteoblasts; Ham and Harris suggested they arise from the endosteal lining of the marrow cavities, and
Bloom suggested they arise from the previous two sources plus from the perivascular undifferentiated mesenchymal cells.

In 1961, Goldhaber proposed the existence of an osteogenic induction factor. He implanted allogeneic neonatal skull bone subcutaneously in a millipore diffusion chamber and found that new bone formation was elicited on the host side of the filter. This new vital bone was derived from the host tissue in response to a diffusible osteogenic inductor coming from the new homograft bone laid down on the inner aspect of the chamber filter. These results support the existence of a cell-free inducing system.

Mann in 1964 described a case report of a fresh autogenous cortical-cancellous bone graft into an infrabony pocket. After four months, a complete fill of the pocket was noted.

Also in 1964, Burwell used autografts, homografts, and homograft-autograft composite of fresh iliac crest bone and marrow. He stated that the two main sources of new bone in the hip marrow grafts are 1) osteoblasts on the surfaces of the grafted bone, and 2) cells contained within the marrow of the graft. The latter is the major source. He concluded that the transplanted marrow produces an osteogenic substance which stimulates osteoblastic differentiation of the undifferentiated mesenchymal cells in the surviving portion of the graft. He proposed that the inducing substance was liberated from the bone marrow when it necrosed after being transplanted. He also
concluded that homografts of fresh marrow-free iliac bone are only weakly, if at all, antigenic.

The following year (1965), Nabers and O'Leary\textsuperscript{39,40} reported the successful use of autogenous bone chips in one and two walled infrabony pockets. The bone chips were obtained from osteoplasty and osteoectomy procedures in the adjacent operative sites. Eight cases, including photographs and radiographs, were presented and all eight cases were successful. This study was not meant as a controlled research study, but merely as a report of several clinical cases.

The manipulation of bony protuberances following extractions and alveolectomies inspired Ewen\textsuperscript{41} in 1965 to apply the same technique in the treatment of infrabony defects, where the bone adjacent to the defect is swaged or moved into the defect. Ross and his co-workers\textsuperscript{42} (1966) noted that this swaged bone procedure consisted in effect of producing a "green stick" fracture. The bone is not remodeled but rather, it is repositioned. The advantage of this procedure is that the vascular supply of the swaged bone is not completely destroyed.

In 1967, Burnette\textsuperscript{43} described the use of an autogenous bone graft in the treatment of one severe interosseous defect between a maxillary lateral and canine. Upon reentry, a complete fill of the defect was noted.
In 1967, Schallhorn described the use of frozen autogenous cancellous bone and marrow from the iliac crest to fill a bifurcation defect. He stated that the iliac crest was not only a very accessible source but was also capable of supplying an unlimited amount of transplant material. The bone was placed in a 50 percent glucose and 50 percent saline media to re-establish a compatible osmotic gradient and therefore prevent lysis. He stated that the freezing preservation technique will preserve the viability of the cells and their potential to undergo differentiation.

In 1967, Urist, Silverman, Buring, Dubuc, and Rosenberg published an article on their concept of the bone induction principle. They stated that the value of a homogeneous bone graft is that it supplies the area with an additional source of inducer substance. The bone inducing principle is either cell bound or closely related to the responding cells and is secreted only during specific stages of cytodifferentiation. It is thought that the protein moiety of bone is included in the bone induction principle. The bone induction principle may be a protein, which itself may or may not behave as a histocompatibility antigen. Hence, while bone induction is suppressed by the immune response of the host, it is possible that it may be an integral part of an autoimmune reaction, in which the inducing protein forms a macromolecular complex with the circulating antibodies of the
recipient. They stated that the bone induction principle is a derivative of a specific protein of the intercellular matrix of bone. They tentatively postulated that the bone induction is an acid insoluble macromolecular protein which is transmitted over short distances but not free in solution in the extracellular spaces. They also reviewed the current concepts of the cell inductive interactions and how they may possibly induce osteogenesis.

In 1968, Hurt described the use of a freeze-dried homogeneous bone in intraosseous defects that were created surgically in dogs. The homogeneous bone was slowly frozen to minus 76°C and was then subjected to a vacuum in order to remove the tissue ice crystals as a vapor leaving the dried tissue with a final moisture content between 1 and 4 percent. The freeze-drying process kills all of the cells in the tissue. Clinically, there was no advantage or disadvantage found in using the freeze-dried homogeneous bone when compared to the usual reattachment procedure utilizing curettage. Histologically, there was convincing evidence of the acceptability of the freeze-dried homogeneous bone in the graft site. No foreign body response was observed at any time in the experiment. Hurt noted that bone was formed on the surface of the freeze-dried homogeneous bone fragments.

Schallhorn, in 1968, reported the use of fresh autogenous bone and marrow in bony crater defects in humans. He proposed
the use of the posterior superior iliac crest as a source of bone when amply oral sites are not available. Upon his 5 month reentry, complete fill of the defect was noted. It was also noted that the crestal level of attachment of the osseous tissue was coronal to that of the former facial and lingual crests.

In 1968, Stallard and Hiatt \(^{46}\) demonstrated new bone and cementum formation around mineralized fragments incorporated in a mucoperiosteal flap. They concluded that bone, dentin and cementum chips, which are left in the wound after periodontal flap surgery, act as a nidus for new bone and cementum formation.

Arrocha, Wittwer, and Gargiulo \(^{142}\) in 1968, implanted processed bovine bone (Boplant) into first premolar extraction sites. They could not demonstrate any osteogenic potential that could be attributed to the Boplant material. The Boplant was not rejected by the dogs; in fact, involucrum formations of the material were seen in the six month specimens.

Stimulated by the work of Nabers and O'Leary, Robinson, \(^{47}\) in 1969, was the first to propose the "osseous coagulum" technique. This technique involves the use of autogenous cortical bone chips from areas adjacent to the bony defect. These chips were mixed with blood and then implanted into the defect. Robinson based his technique on two assumptions: 1) that the smaller the particle size of the donor bone, the more certain are its resorption and replacement, and 2) that mineralized fragments can induce osteogenesis.
Cushing,\textsuperscript{50} in 1969, reviewed the literature concerning autogenous red marrow grafts. He stated that it stimulates osteogenesis consistently when transplanted. The great potential of this tissue to form bone appears to be due to the availability of source cells lining the vascular sinusoids which have the propensity to differentiate into osteoblasts. This differentiation occurs as a result of an inductive signal seemingly initiated by products of the necrosing marrow and bone.

In 1971, Rivault\textsuperscript{48} and in 1972, Coverly\textsuperscript{49} described the sequential histologic healing of the osseous coagulum technique of creating periodontal defects. Both studies concluded that the healing of the defect with the graft was more rapid than the control defect. It was also shown that the graft underwent necrosis before the initiation of osteogenesis. Once started, the osteogenesis was seen on the surface of the graft particles and on the walls of the defect. Both studies used the same method of defect creation. Once the mucoperiosteal flap was reflected, a bur was used to cut a two-walled defect. After the flap was sutured back in place, a wooden irritant was placed into the defect. The presence of the irritant allowed the bacteria and their toxins to enter the defect. Histologically and clinically, the defect appeared as a chronic periodontal osseous lesion.
In 1970, Schallhorn, Hiatt, and Boyce performed 182 iliac bone and marrow transplants on 52 patients. A large majority of these autografts were frozen. They found that the two-walled osseous defects filled completely to the height of the adjacent crest, and in some instances formed new bone supracrestally. The one-walled osseous defects and furcation defects either partially or completely filled in with new bone. They stated that there were several consistent findings observed as the study progressed: 1) considerable sequestration occurred in many of the implant sites, 2) many cases required considerably longer post-operative time to show radiographic appearance of maturation, 3) early pocket closure (1 to 2 months), 4) the inductive effect of the implant at a site removed from the immediate area of implantation, and 5) root resorption in some of the treated areas. They also stated that the success of the autogenous bone and marrow grafts lies in the proper preservation of cellular structure until implanted. The ensuing cellular breakdown and release of some inducing substances may then be the key to osteoblastic differentiation from surrounding cellular elements.

In 1970, Seibert reported the successful use of a fresh autogenous hip marrow and cancellous bone in an intrabony defect. He stated that the error of over filling the bony defect with the
graft material to such an extent that the soft tissue flap cannot cover and seal the area of the implant should be avoided. Any donor bone and attendant cellular elements exposed to the environment of the oral cavity will become non-vital above the area where it is not protected by the fibrin seal of the clotting wound and nourished by the plasmatic circulation of the surrounding tissue. The osteoclastic response marshalled to resorb the exposed non-vital bone and repair the dento-osseous margin may well endanger the contiguous cemental and dentinal surfaces to unwanted resorption and delayed repair.

Rosenberg in 1971, reported the use of fresh autogenous marrow and cancellous bone from intraoral sources—mandibular retromolar area, maxillary tuberosity, edentulous ridges, and buccal ledges and plates. He observed clinically and radiographically 400 lesions treated with the autograft and reported successful results. He stated that the greatest limitation to such a treatment was the inadequate amount of graft material available to fill multiple lesions.

In 1971, Hiatt and Schallhorn reported the use of frozen human allografts of iliac cancellous bone and marrow in osseous defects. The bone and marrow were obtained from "living cadavers." No cementogenesis was evident but osteogenesis occurred on several bony trabeculae. They stated that either the graft retained viability of the osteocytes while the marrow was replaced with
repair tissue or the trabeculae are artifacts of autogenous bone incorporated into the site during instrumentation.

In 1972, Register et al. implanted allogeneic dentin matrix into a stab wound in the gingival papillae. They observed that the dentin matrix, which was not rejected, induced the undifferentiated mesenchymal cells of the host to differentiate into osteoblasts and form mature bone. The 90 day specimen revealed a small marrow cavity in the center of an ossicle.

Lane et al., in 1972, compared fresh autogenous and freeze-dried homogeneous bone grafts in the mandible of rhesus monkeys. The inferior border of the mandible was used as the donor and recipient sites. The radiographic, clinical, and histologic results indicate that freeze-dried bone is an acceptable graft material. They stated that if the homograft was slow to be replaced, there was a tendency for the autograft in the same animal also to repair slowly. This indicates the desirability of controlling and comparing the autograft and homograft in the same animal whenever possible.

Narang et al., in 1972, employed grafts of decalcified allogeneic bone matrix to add height to the alveolar ridges in dogs. The grafts were not rejected and new bone formation was seen at the site of implantation. This study showed that the allograft is useful for ridge augmentation.
In 1973, Morris compared fresh and frozen autogenous hip marrow grafts in rhesus monkeys. He surgically created the two-walled osseous defects with a bur and placed a wooden irritant into the lesion to create a chronic wound which is similar to chronic periodontitis. He found the sequential healing of the fresh and frozen autografts to be similar. No difference was found between the osteogenic potential of either autograft. Both induce the differentiation of the undifferentiated mesenchymal cells into osteoblasts which in turn form new bone. He did note some external root resorption above the base of the defect in the fresh autograft.

In 1973, Hiatt and Schallhorn performed 166 intraoral transplants on 40 patients. The maxillary tuberosity, healing sockets, and edentulous areas were used as donor sites. An average fill of 3.44 mm was obtained which compares favorably to iliac autografts. They stated that while fill of crestal, facial, and furcation defects was noted, it did not appear to be predictable when compared with iliac autografts.

In 1973, Dragoo as well as Dragoo teamed with Sullivan reported on the use of fresh autogenous iliac bone and marrow in humans. They noted that the intraosseous defects not only filled but there was a 0.70 mm average increase in the crestal bone height. True reattachment was achieved in that new bone, PDL, and cementum was formed. Cementogenesis was noted in all sections
as new cellular cementum is deposited. They also noted some root resorption in some of the cases which they attributed to chronic inflammation of the adjacent gingiva. Once the inflammation is alleviated via curettage, they noted that root repair is seen. However, further root resorption was seen at a later date.\textsuperscript{59} They stated that there are three possible etiologic factors in the resorptive process of the roots: 1) the fresh marrow with undifferentiated mesenchymal cells may contribute to the decalcification of the root surfaces, 2) the resorption of sequestered bone near the epithelial attachment may initiate the breakdown of the adjacent tooth surface and, 3) the proteolytic enzyme activity of PMNS, macrophages, and other chronic inflammatory cells may also contribute to the resorption.

In 1974, Boyne\textsuperscript{60} utilized fresh autogenous iliac crest cancellous bone and marrow to produce a complete osseous union of simulated alveolar and palatal clefts in young rhesus monkeys. Histological evidence was presented which demonstrated that the grafted bone underwent a remodeling process to form an apparently normal trabecular pattern and alveolar cortical surfaces of lamellated bone. The control defects, which did not receive a bone graft, did not show regeneration of new bone in the clefts.

Malevich et al.,\textsuperscript{61} in 1974, used boiled, compressed homograft bone, formed to the shape of a screw, to fix mandibular fractures instead of using metal bone pins. The course of
consolidation of the fractures and the fate of the screw fixers was studied in 32 dogs. Homobone screws were found to resorb slowly and were completely replaced by the newly-formed bone by 18 months. They stated that no inhibitory effect was produced on the consolidation of the fractures. They also used the screw homobone to treat linear, comminuted, and spiral fractures of the mandible in 55 clinic patients with good results.

Libin, Ward, and Fishman, in 1975, reported three cases in which decalcified, lyophilized bone allografts were used to treat severe periodontal defects in humans. They reported that there were no signs of the graft material being immunologically rejected by the host. They indicated that it was possible to restore hard and soft tissue attachment in areas of severe periodontal destruction.

Research seems to point to autogenous hematopoietic marrow and cancellous bone as being the most optimal graft material for the osseous grafting procedures. However, although allogeneic hematopoietic marrow and cancellous bone yield less bone regeneration, it does offer the advantages of eliminating a second procedure and the resultant patient's hip discomfort.

Several theories of the mechanism of repair of bone grafts have been proposed but, as yet, it is still unclear. This is especially true when it refers to the allograft. With this study,
additional histologic information about the repair phenomena of both fresh and frozen allogeneic iliac bone grafts in chronic periodontal osseous defects should be provided. By providing a better understanding of the repair phenomena, our study should give new insight into the clinical applications of allogeneic bone grafts.

B. TISSUE PRESERVATION

A classic slow-freezing technique, using glycerol as a cryoprotective agent, was described by Smith, Polge, and Parkes\textsuperscript{74,75} to preserve various mammalian tissues. They successfully preserved bull spermatozoa at \(-79^\circ\) C. When thawed at \(40^\circ\) C, the spermatozoa were found to be motile and capable of full function. Also, no alterations in the genetic qualities were seen as a result of the freezing. Smith\textsuperscript{76} stated that a wide variety of living cells and tissues could be stored for long periods of time at low temperatures, using glycerol as a cryoprotective agent. These cells and tissues can, at a later date, be utilized in diagnostic, therapeutic and experimental medicine.

Pegg\textsuperscript{,77-79} described a successful controlled freezing technique. He preserved human marrow at \(-79^\circ\) C by slowly freezing and rapidly thawing. He utilized 15\% glycerol as a
cryoprotective agent. He noted, however, that cellular deterioration was evident at this temperature, and proposed that, for longer periods of storage, a lower temperature and the cryoprotective agent, dimethyl sulfoxide (DMSO), should be used.

Boyne and Yeager compared the osteogenic potential of fresh and frozen autogenous marrow and found them to be similar. They utilized a controlled freezing technique employing liquid nitrogen (-196°C) and dimethyl sulfoxide as a cryoprotective agent. They noted that frozen marrow would stimulate host bone tissue to undergo osseous proliferation. Also, no clinical difference was noted between the osteogenic potential of fresh and frozen marrow tissue.

Malinin compared different cryoprotective agents and temperatures for freezing human bone marrow cells. He found that after three years of storage in dry ice (-79°C) using glycerol as a cryoprotective agent, almost all of the cells were destroyed. He noted nuclear vacuolation, pyknosis, and karyolysis. On the other hand, after three years of storage in liquid nitrogen (-196°C) using dimethyl sulfoxide as a cryoprotective agent, the cells were found to be intact and viable.

Rubinstein and Trobaugh described a technique for the preservation of hematopoietic tissue. They utilized glycerol as a cryoprotective agent and froze the tissue to -196°C with
liquid nitrogen. Using the electron microscope, a description of the results of this freezing technique was given. They noted the presence of the viable "presumptive stem cells" in the bone marrow suspension. Also noted was the ability of the frozen and thawed hematopoietic tissue to repopulate similar to the fresh marrow. Barkin and Newman also gave an ultrastructural description of the results of this controlled freezing technique. They noted various degrees of cellular deterioration but also noted that the reticular and blast cells remain viable after programmed freezing.

In 1956, Krone, in his classic article, was the first to describe the use of frozen bone in the treatment of intrabony pockets. The bone was frozen in a 1:1000 merthiolate solution. The freezing technique employed was not described in detail. Eight of the 13 cases were successful.

Schallhorn, Hiatt, and Boyce reported on the use of frozen cancellous bone and hip marrow for the correction of periodontal osseous defects. They described three different methods of storing cancellous bone and marrow depending on how long the interval existed between obtaining and placing the graft material. The direct technique of storage, which is used to store the graft for 1-3 hrs, involves storing the graft in Ringer's solution at room temperature. The short term technique, which is used to preserve the graft for one week, involves the use
of a minimum essential media plus 5 to 15% glycerol and storage at 4°C. The long term technique, which is used to store the graft for several months, involves the use of a minimum essential media plus 25% glycerol and storage at -79°C. This study reinforced the concept that frozen bone and marrow keeps its potential for osteogenesis. They theorized that the success of the graft depends on the proper preservation of its cellular structure.

C. CRYOINJURY

In the past quarter of a century, research in the area of cryobiology has received considerable attention. Preserving viable cells and their potentials for function by freezing has been accomplished repeatedly since Polge et al. cited the use of glycerol and Ashwood-Smith introduced the use of dimethyl sulfoxide (DMSO) as cryoprotective agents. Cellular deterioration and the cryoprotective mechanism have proved to be the greatest stumbling blocks to further progress in cryobiology.

The concentration and nature of the cryoprotective agent and the rate and mechanism of freezing and thawing were all implicated by Meryman as factors influencing the ability of a tissue to retain its structure and function during the freezing process. The following processes were mentioned as possible causes of cellular deterioration: the effect of osmotic stress on
membranes, the catabolic activity of lysosomal enzymes causing cryoinjury, formation of ice crystals in the cells, movement of electrolytes into the cells resulting in a greater intracellular concentration, different vapor pressures resulting in water resorption and redistribution, and the toxic effects from pretreatment with cryoprotective agents.

The freeze-etching technique was utilized by Steere to study the formation of crystal of ice in bacteria, monkey intestinal epithelium, red blood cells, and dog and mice heart muscle cells. He observed that increasing glycerol concentrations up to the 40% level resulted in decreasing the amount of ice crystal formation. The probability of ice crystal formation for several tissues has been formulated by Mazur.

Farant et al. reported that glycerol, when used as a cryoprotective agent, decreased intracellular ice crystal formation. As less ice crystals form during the freezing process, there is less danger of an intracellular increase in the electrolyte concentration. They theorized that high electrolyte concentrations during freezing and swelling of the cells due to an increase in the osmotic pressure during thawing are responsible for the cell damage during the slow freezing process.

Sherman used mouse skin autografts to compare the survival of frozen-thawed versus unfrozen-control autografts. He utilized
both glycerol and dimethyl sulfoxide as cryoprotective agents varying their concentration from 5 to 15%. It was found that during the pretreatment with the cryoprotective agents, cells were damaged. In some cases, the damage was extensive enough to cause the loss of some of the skin autografts. He noted that the cellular damage was due to the cryoprotective agents and not the freeze-thawing process. He also noted that this cellular damage was proportional to the concentration of cryoprotective agents due to the increased toxicity.

Barchi et al. theorized about the cell electrolyte concentration being a mediator in cellular deterioration during freezing. They noted that the slow freezing technique caused cellular dehydration which was secondary to ice crystal formation in the extracellular areas. This dehydration of the cells and the crystallization of slightly soluble buffer salts results in an increase in the intracellular electrolyte concentration and a shift in the acid-base balance. The change in the electrolyte concentration and the pH can produce denaturation of lipoproteins. Since cell membranes are lipoprotein complexes, they can be damaged via denaturation as a result of the freezing process and its consequences.

Meryman, studying frog muscles, noted that glycerol caused a sharp fall in the membrane resting potential. When the tissue was placed into an isotonic media, the membrane resting potential
did not return to normal. For cryoprotective agents to protect, they must be in high concentrations. The problem comes with the increased toxicity of higher concentrations.

Earlier, Meryman theorized that cellular damage was not caused by an increased intracellular electrolyte concentration; instead, the extracellular solutes which could not pass through the cell membrane increase in concentration and produce an elevated osmotic pressure. In 1971, he studied the freezing injury to human red blood cells. He found that the osmotic loss of the cell liquid phase and reduction of cell volume which results, causes an increased stress on the cell. The increasing stress reaches a point when the integrity of the cell membrane is lost and extracellular materials begin to ingress. He noted that the limiting stress appears unrelated to the concentration of the extracellular and intracellular solvents and solutes, but related to the proportion of the cell liquid phase removed. Meryman elaborated on this theory when he stated that this osmotic stress is the main cause of cryoinjury during the freezing process. He proposed the theory that the osmotic stress is due to intracellular resistance to unrestrained reduction of cell volume causing an osmotic gradient across the cell membrane. He called the theory the "minimum cell volume" hypothesis. It states that membrane damage results from osmotic stress imposed on the cell membrane when the cell volume is decreased beyond a minimum
tolerable size.

Persidsky, using rat bone marrow cells, studied the effects of cryopreservation to see if lysosomal enzyme activation was a factor in the cryoinjury mechanism. He compared the degree of cell viability of frozen bone marrow cells treated with trypan blue, which inhibits the lysosomal enzymes, with frozen untreated bone marrow cells, which served as a control. He found that bone marrow cells treated with trypan blue recovered much better after freezing and thawing and he theorized that it was due to the trypan blue inhibition of lysosomal enzymes. Lysosomal enzymes are known to cause cytolytic activity. Persidsky further theorized that the freezing and thawing process could trigger lysosomes to release their enzymes and thus cause cellular damage. Therefore, cryoinjury of cells, especially those with high lysosomal content, may be caused directly by the catabolic action of lysosomal hydrolases.

Litvan proposed a different hypothesis to explain cryoinjury. He assumed that intracellular water remains a liquid when the temperature drops below 0°C, thus causing an increase in the intracellular pressure due to the fact that water has a greater vapor pressure than ice. Below 0°C, the extracellular water forms ice and therefore, its vapor pressure is less than it is intracellularly. As the temperature decreases, this vapor pressure differential increases. This situation results in the
gradual release of bound water and a redistribution of this water because of the existing non-equilibrium state. Therefore, the slow cooling rates and the resultant dehydration of the cells causes a rupture of the cell membranes. Litvan explained that cryoprotective agents work by decreasing the rate of water distribution and by decreasing the vapor pressure differential.

Litvan\textsuperscript{91} also explained the thawing process. During a rapid thawing, there is an incomplete disappearance of ice crystals in some cells while some cells thaw completely. This creates a deleterious vapor pressure difference and a rapid water diffusion which results in cellular injury.

D. IMMUNE RESPONSE

In 1955, Bonfiglio et al.\textsuperscript{97} used fresh and frozen homografts of bone to repair ulnar fractures in rabbits. They found that animals immunized with bone extracts with subsequent frozen bone homografts produced a low antibody titer demonstrated by complement fixation tests. These animals showed a markedly diminished inflammatory response to the homograft. On the other hand, if a homograft is placed in an unimmunized animal, a greater inflammatory response is noted. They also noted that frozen homografts exhibit a less intense initial inflammatory response than fresh
homograft; but the inflammation persists much longer due to the
delayed vascular invasion and resorption of the graft.

Enneking, in 1957, studied allogeneic and autogenous trans-
plants in rats. He noted a variation in the inflammatory response
to different allogeneic transplants and attributed this variation
of host response to the degree of inbreeding of the rats used in
the experiment.

Zeiss et al. in 1960, using rats, studied autografts and
allografts of cortical bone implanted into the subdermal tissue.
They found many of the surface osteocytes in the allograft sur-
vived up to 250 days. They explain this survival of osteocytes
in that host lymphocytes, carrying antibodies, were unable to
penetrate into the compact bone of the graft, which means that
the bone acts as a protective barrier against osteocyte destruc-
tion.

In 1959, Curtiss et al. described the reaction of the host
to a homograft. They stated that the graft tissue releases an
antigenic substance which is picked up by the host-graft bed
lymphatics and carried to the regional lymph nodes. In the lymph
nodes, the antibodies are produced against these antigens. These
antibodies are attached to lymphocytes, not circulating antibodies.
The actual antigenic matter, they proposed, is a lipopolysacchar-
ide which is separable from the DNA-protein complex. They also
stated that this antigenic material is of cell nuclear origin.
Also, in 1959, Billingham\textsuperscript{101} stated that there is no cellular or tissue specificity involved in tissue transplantation immunity. He stated that this is suggested by a body of evidence showing that the sensitization evoked in a recipient by one type of tissue or cellular homograft is fully effective against subsequent homografts of other types of tissue from the same donor. He also noted that the antigenic substances responsible for eliciting homogeneous reactions are determined by multiple dominant Mendelian genes, known as histocompatibility genes. There are at least 15 different genetic loci on the chromosomes for these genes.

Hammack and Enneking,\textsuperscript{102} in 1960, described the host rejection reaction in rats. They noted that by the eighth or tenth day, while the autograft continues to proliferate, the homograft evokes an antigen-antibody inflammatory response that obliterates the vascular supply to the periosteal proliferation causing necrosis.

In 1960, Heslop \textit{et al.}\textsuperscript{103} observed that there is a decrease in the number of lymphocytes and plasma cells around a homograft at 35 days as compared to 14 days. The reasons for the diminution of the inflammatory cell infiltrate is uncertain. The differences in the proportions of surviving homologous osteocytes, which presumably excited the reaction, were not sufficiently great to explain the decrease in cells. They suggested two
possible explanations. First, there is an exhaustion of the recipient's reticuloendothelial system—this is not very probable. Secondly, there is a change or decrease in the antigenicity of the grafted cells, in other words, an adaptation. They believe the latter to be the more plausible.

Also, in 1960, Nisbet et al.\(^{104}\) compared cortical bone autografts and homografts to which immunologic tolerance had been induced 24 hours after birth by injecting donor spleen cells. They noted that the reluctance of the host to vascularize the homogeneous cortical bone grafts from their original donor was overcome and the inflammatory reaction to the antigenic stimulus of foreign osteocytes was suppressed. They also noted that the host is able to replace the foreign cortical bone graft with new bone similar to the autograft.

Medawar\(^{105}\) and Burnet\(^{106}\) in 1961, defined immunologic tolerance as a state of indifference or non-reactivity towards a substance that normally is expected to excite an immunological response. They stated that tolerance is a systemic alteration of the host and not an antigenic adaptation of the grafted cells. Also, tolerance is not an all or none phenomenon, but rather, found in degrees. Tolerance is not necessarily permanent; the presence of the antigen is needed as a continual reinforcement.
In 1966, Billingham\textsuperscript{107} reported on the clinical approach to tissue transplantation. He stated that the destruction of a homograft is an immunological phenomenon, the outcome of a highly specific attack on the part of the host against foreign genetically determined cellular isoantigens known as transplantation antigens. Since the rejection reaction is immunologic in nature, immunosuppressive drugs can be used with excellent results. The dosage of the drug is important in that it must suppress the rejection reaction but not endanger the host's immunologic defense mechanism.

McLean and Urist\textsuperscript{108} in 1968, stated that the current concept of antigenic activity is that when fresh living allogeneic tissues are transferred, antigens pass directly via the host lymphatics to the regional lymph nodes, where antibodies are formed; the spleen also reacts to antigens reaching it through the blood, but this response is quantitatively of lesser importance. The antigens in allogeneic bone that lead to the production of antibodies have not been accurately identified; they are formed by and reside in or on cells; their formation is under the genetic control of the nucleic acids of the cells. For as long a transferred allogeneic bone proliferates, it continues to form antigens and thus invokes the immune response of the host. The antibodies produced in the presence of an allogeneic graft are mainly carried by cells (lymphocytes) produced in the regional lymph nodes.
The participation of humoral antibodies in the immune response to allogeneic grafts is not excluded, although their effects on grafts are negligible.

Hiatt, in 1970, studied the difference in healing of allografts of cancellous bone and marrow with and without the use of the immunosuppressant antilymphocyte globulin (ALG) in periodontal defects in dogs. ALG blocks the immunologic activity of lymphoid cells. He found no difference in the healing between the allografts with or without ALG. He stated that some tissues are privileged and do not elicit the usual immunologic response when grafted in humans; these tissues are referred to as non-viable structures, i.e., cornea, dense bone, and blood vessels. Hiatt proposed the initial environment and anatomy of the healing periodontal lesion might permit the allograft to reside in prepared pockets, relatively unnoticed antigenically. Until the connective tissue and epithelial attachment are constructed, it must be assumed that the exudation of crevicular or inflammatory fluid continues from the surrounding vessels and through the pocket. While this outward flow may not prevent foreign protein from penetrating the surrounding vascularity, he speculated that the antigenic stimulus would be limited until a hook-up of the circulation occurred between the graft and the host. If one to two weeks is needed for initial repair and the beginning of vascularization of the graft plus one to two weeks more for antibody
formation against the allograft, then it is possible that the initial collagen repair is complete before any rejection might occur.

In 1971, Hiatt and Schallhorn reported on the use of iliac cancellous bone and marrow allografts in humans. None of the grafts were rejected. Sherk and Nicholson reported on the use of allogeneic bank bone in leg fractures. They stated that bone from the same donor may be tolerated several times by the same recipient without fear of rejection.

Congdon, in 1971, mentioned several immunosuppressive agents which will help prevent the rejection of bone marrow allografts, i.e., azathioprene, cyclophosphamide, antilymphocyte serum, and whole body ionizing radiation.

Merin et al., in 1973, studied the effect on healing of bone and marrow isografts and allografts in rats. They found a delayed bone regeneration in the allografts due to the immunologic rejection. The majority of the rejection reactions occurred between the second and fourth weeks. Bone regeneration was also less in the allografts than in the isografts and about the same as the control site receiving no graft. They concluded that some degree of histocompatibility between donor and host is necessary.

Also in 1973, Summerlin et al. studied allogeneic skin grafts. They found the fresh were rejected in both humans and mice, but cultured skin allografts were not rejected in either
humans or mice. The cultured allografts did not elicit a T-cell response assayed via lymphocyte cytotoxicity tests. They proposed some possible factors that should be considered in an explanation of the acceptance of cultured allografts: 1) the grafts were notably hypocellular when they were transplanted; 2) antigen masking or modification must be considered, since it has been shown that histocompatibility antigens are not lost; and 3) passenger lymphocytes rapidly disappear in culture (24-36 hours).
CHAPTER III

MATERIALS AND METHODS

A. EXPERIMENTAL DESIGN

Four adult rhesus monkeys (Macaca mulatta), one female and three males, were utilized in this study as the experimental models. Upon arrival at the research center, the animals were examined by a veterinarian and found to be in good health. The animals remained in good health throughout the experimental period of time. The animals were given 10 days, prior to the beginning of the research project, to become acclimated to the new environment and the diet that was to be used during the experimental period. Each animal possessed a full complement of teeth with similar periodontal findings. Plaque, calculus, and food debris accumulations were found on all the teeth. All of the monkeys presented with a marginal chronic gingivitis. The interdental papillae and the marginal gingiva were edematous, magenta in color, and slightly boggy (Figure 1). The depth of the sulcus around the teeth varied between one and three millimeters.

The maxillary and mandibular quadrants on the right side were utilized as experimental sites. Each quadrant served as a site
for two-walled surgically created defects. One of the defects in each quadrant was corrected with a fresh and one with a frozen allogeneic iliac crest bone and marrow transplant thirty days after the defect creation. A third two-walled defect was treated in three of the quadrants and these did not receive a bone graft but served as a control site. These defects were created with curettage alone at the same time the bone grafts were placed.

At the time of sacrifice of the animals, both a fresh and a frozen transplant were obtained for the post-operative healing periods of 0, 3, 7, 14, 21, 28, 42, and 56 days. Control defects were obtained at 7, 21, 42 days, post-operatively.

Fifteen minutes prior to any surgical procedure, the monkeys were given intramuscular injections of 8 mg of Sernylan* (Figure 5) for sedation. If at any point during the surgical procedure, the sedation became too light, an additional 5 mg of Sernylan was given intramuscularly. Once sedated, the monkey was removed from its cage and brought to the procedural room. The monkey was positioned on the surgical table in such a position so as to maintain a patent airway. Aseptic procedures were used throughout the project.

**B. GENERAL PREPARATION**

Full buccal mucoperiosteal flaps were reflected in order to

*Parke, Davis, and Co., Detroit, Michigan*
visualize the alveolar crest during the creation of the defects and the repair of the lesions (Figure 2). An intrasulcular incision to the alveolar crest was utilized extending from the mesial of the cuspid to the distal of the second molar. A number 15C Bard Parker blade was used. A scalloped incision was used in order to preserve as much of the papillary tissue as possible. The papillae were preserved in order to get primary coverage over the bone grafts. A wax spatula was used as a periosteal elevator, because of its small size, to reflect the buccal mucoperiosteal flaps. Tissue tags were scissored away from the internal surface of the flaps. Gracey curettes were utilized to remove all granulation tissue and to plane the roots.

C. PREPARATION OF DEFECTS

The sites chosen for the osseous defects were the interproximals adjacent to the second bicuspid. These areas were easily accessible. Also, there was a sufficient amount of bone present to allow for the creation of the two-walled defects. The interproximals between the first and second molars were utilized for the osseous defects that served as the control sites for the 7, 21, and 42 day specimens.

The type of osseous defects utilized in this project were of the two-walled variety. A 701 tapered fissure bur was used in a
slow speed dental handpiece to remove 3 mm of alveolar crest bone. A Michigan periodontal probe was utilized to insure a constant depth of 3 mm in all of the defects created. The osseous defect was created along the distal root surface of the aforementioned teeth. The resulting osseous defects consisted of a lingual and distal wall of bone, a mesial wall of cementum and/or dentin and, no buccal wall (Figure 3). While removing the bone during the creation of the defects, isotonic saline was used as a coolant to avoid temperature increases which could damage the bone.

D. INTRODUCTION OF CHRONIC IRRITANT

A wooden toothpick approximately 6 mm in length was introduced into each osseous defect to serve as a chronic irritant (Figure 4). The irritant was introduced into the defect from the buccal aspect after the flap had been reapproximated and sutured into place. Once in the defect, the irritant was revolved into a position parallel to the tooth with the occlusal part of the irritant wedged into the gingival embrasure just below the contact area. The irritant served as a source of chronic irritation to the osseous defect. In addition, it allowed the ingress of bacteria and bacterial toxins. This procedure enabled the osseous lesions to simulate those found in human periodontitis.
When the buccal mucoperiosteal flap was reapproximated to its original position, it was sutured into place with 5-0 silk sutures using the interrupted interproximal suturing technique. This suturing technique provided additional stabilization of the chronic irritant.

E. **POST-OPERATIVE CARE**

Prior to the initiation of any surgical procedure, the monkey received a prophylactic injection of an antibiotic. An intramuscular injection of 600,000 units (3 cc) of Combiotic* was given. Combiotic is a combination of penicillin and dihydrostreptomycin. Post-operative instructions were written up specifying that the animals receive no food for the first 24 hours postsurgically, a soft diet for the following 2 days, and a normal diet thereafter.

F. **REMOVAL OF IRRITANT**

Seven days following the creation of the osseous defects, the animal was sedated with an intramuscular injection of 8 mg of Sernylan and the sutures and wooden irritants were removed. All clinical observations were recorded.

*Pfizer Company
G. PROCUREMENT OF GRAFT TISSUE

The technique used to obtain the iliac crest graft material is the same as that described by Dragoo and Irwin,\textsuperscript{115} in 1972, and by Morris,\textsuperscript{6} in 1973. Seven days prior to the scheduled time for the correction of the defects, iliac crest material was obtained for preservation by controlled freezing. After the monkey was sedated, the operative site overlying the anterior superior iliac spine and crest was shaved, scrubbed with surgical soap, and Betadine antiseptic germicide* was applied to the skin (Figure 5). The area was then isolated with sterile towels.

Finger palpation was used to find the anatomic landmarks. A 5 mm incision to the bone was made with a number 15 Bard-Parker blade along the anterior superior iliac crest at the area of the anterior superior iliac spine. The surrounding tissues were dissected and the cortical bone of the iliac crest was exposed.

A Turkel Trephine needle** was then utilized to obtain multiple cores of cancellous bone and marrow from the iliac crest (Figure 6). The outer needle was carefully aligned to follow the medial-posterior inclination of the ilium, and was then rotated slightly to penetrate the periosteum and cortical bone. The inner trephine needle was then inserted into the outer needle, and with slight pressure and rotation, it penetrated the remaining cortical bone and entered

*Purdue Frederick Company
**Turkel Instrument Company, Detroit, Michigan
cancellous bone. The inner needle, which then contained bone, was slowly withdrawn while the outer needle remained embedded in the cortical bone of the anterior crest. An inner needle stylet was inserted into the inner needle trephine to remove the core of cancellous bone into a small freezing vial containing Hank's balanced solution with 12% glycerol (Figure 7). The technique was repeated until the desired number of cores had been obtained, usually 2 or 3, and then the outer needle was withdrawn. The wound was closed by suturing in layers. The innermost layers were sutured with triple-0 resorbable cat gut while the outermost layers were sutured with 3-0 silk suture material (Figure 8). At this time, the Betadine solution was again applied to the surface. The same postoperative antibiotics were administered as described previously, and the animal was returned to the cage. The silk sutures placed in the hip were removed in seven days following their placement (Figures 9 and 10).

The vials containing the bone cores in Hank's balanced salt solution and glycerol were immediately placed in an insulated container, packed with ice (0°C), and transported to Rush-Presbyterian St. Luke's Medical Center, Chicago, for controlled freezing. These specimens later served as the frozen allogeneic bone graft material. At the time of defect correction, the fresh allogeneic bone graft material was obtained from a contralateral iliac crest utilizing the same procedure.
H. FREEZING AND THAWING THE TISSUE

Controlled freezing of the specimens was accomplished by placing the freezing vials into a Linde BF-4-1 freezing chamber (Honeywell Laboratory Products) and flooding the cooling chamber with vaporized nitrogen. The tissue was frozen to -12°C at a rate of 3°C per minute. Immediately following the release of the latent heat of fusion, the tissue was frozen to -100°C at a rate of 2°C per minute. The freezing vials were then rapidly transferred to a liquid nitrogen storage tank (-196°C) where they were maintained for seven days.6

One hour prior to the surgical correction of the osseous defects, the frozen vials were placed in an insulated container, packed with frozen CO₂ (-79°C), and transported to Loyola University. Shortly thereafter, when the surgical procedure had progressed to the point where the bone graft was indicated, the vials were immersed in a 40°C water bath and thawed for 2 minutes.

I. DEGLYCEROLIZATION

After thawing, the vials were opened and the contents poured into separate sterile beakers. Immediately following, at 2 minute intervals, the following amounts of sterile 35% glucose
and 6% Dextran solution were added to each beaker: 0.5 cc glucose; 0.75 cc Dextran; 2.50 cc Dextran; 3.00 cc Dextran and 2.25 cc Dextran.

After deglycerolization, the cancellous bone and marrow cores were immediately utilized in the bone graft procedure.

J. CORRECTION OF DEFECTS

Thirty days after the surgical creation of the two-walled osseous defects, they were corrected with a fresh or frozen allogeneic bone graft, or by curettage. An allograft is graft material from a donor within the same species.

Fresh cancellous iliac bone had to be obtained just prior to the intraoral surgery. Procuring the fresh bone cores was achieved in an identical manner as that described in section G of this chapter. A contralateral hip served as the donor site. The fresh bone cores obtained were placed in a beaker containing sterile isotonic saline solution as a holding media.

The same surgical procedures and considerations outlined previously were used to expose the osseous defects for surgical correction. A buccal mucoperiosteal flap was reflected, the root surfaces of the teeth thoroughly planed, and the granulation tissue in the defects and surrounding areas meticulously removed by curettage.
The frozen bone cores were then thawed and deglycerolized according to the protocol described. Both fresh and frozen cancellous bone cores were therefore ready for intraoral grafting. A #15 Bard Parker blade was used to slice the cancellous bone cores into small sections (approximately 0.5 mm x 0.5 mm), for this would facilitate placement of the graft material and assure a more complete filling of the defects.

After inspection of the defects, the cancellous bone and marrow was placed into the defects. A standardized procedure was followed for all grafts which consisted of the placement of fresh bone material into the distal of first bicuspid defect and frozen bone material into the distal of second bicuspid defect. The cancellous bone was firmly packed into the defects until they were filled, but no attempt was made to overfill them.

The control defects underwent the same procedure as the graft recipient sites; however, after they were curetted of all granulation tissue, they received no graft.

After the surgical correction of the defects were completed, the mucoperiosteal flap was reapposed, and firmly fixed over the osseous lesions with interproximal interrupted sutures of 4-0 silk (Figure 11). The same postoperative antibiotics and diet supplements as previously described were given.

Seven days following the correction of the defects, the animal was again sedated and the sutures were removed. The monkey
was then maintained until the next procedure, or until the scheduled sacrifice.

K. COLLECTION OF SPECIMENS

Following sedation with Sernylan, the animals were given an intraperitoneal injection of a lethal dose of Totaltox*. Block sections that included the experimental sites were then obtained. After cutting each section free from the surrounding structures with an electric Stryker saw, it was washed with water, tagged for identification, and placed into a jar containing 10% formalin for fixation. The animals were handled according to the standards established by the National Society for Animal Research.

L. PREPARATION FOR HISTOLOGIC EXAMINATION

Following adequate fixation (2 weeks), each specimen was decalcified in formic acid and sodium citrate (4 weeks), trimmed, embedded in paraffin, sectioned at 7 microns in a transverse bicipholingual plane, and stained with hematoxylin and eosin. On a few selected sections, a silver stain was done to verify the presence of collagen in the new periodontal ligament. The slides from each experimental site were studied, and a representative histologic section was selected for detailed histologic analysis.

*Chicago Veterinary Supply, Chicago, Illinois
CHAPTER IV

FINDINGS

A. CLINICAL OBSERVATIONS

All of the monkeys presented with a marginal chronic gingivitis. The interdental papillae and the marginal gingiva were edematous, magenta in color, and slightly boggy (Figure 1). The sulcus depth around the teeth varied between one and three millimeters. Seven days following the creation of the osseous defects and placement of the irritants, the gingiva showed a marked increase in the amount of inflammation. The marginal and papillary gingiva was erythematous, edematous, and bled easily upon light probing. At the time when the osseous defect underwent correction, thirty days following the creation of the osseous defect, there was a decrease in the amount of inflammation noted, compared to the seven day postoperative picture. The papillary and marginal gingiva still demonstrated edema, erythema, a boggy consistency, and hemorrhage upon light probing. The pocket depth in the area of the created defect measured five millimeters.

The osseous topography at the time of the defect correction, thirty days after creation, demonstrated additional bone
resorption. The buccal cortical plate showed more resorption mesial and distal to the defect creating a V-shaped defect.

Seven days following the correction of the defects, the marginal gingiva was edematous, very erythematous, and boggy in consistency. The papillae also demonstrated these same inflammatory characteristics. The gingival tissue over the control defects show less inflammation at this time interval. By the fourteenth day post-operative, the gingival tissue appeared almost normal and continued to improve in appearance as the healing process continued.

B. HISTOLOGIC OBSERVATIONS

1. INTRODUCTION

The epithelium, early in the experiment, demonstrated marked inflammatory changes which could be attributed to several factors: the surgical insult, the chronic gingivitis that was present, and the reaction to the presence of the allograft. Later in the experiment, the epithelium appeared to be within normal limits (Figure 12).

The connective tissue changes were characterized by inflammation first and this was followed by repair. The inflammatory changes consisted of vasodilatation, edema, and inflammatory cell infiltration. The changes associated with repair consisted of fibroblastic and endothelial
proliferation and organization of gingival fibers.

The alveolar bone changes, especially at the crest, consisted of repair and remodeling (Figures 27 and 42).

The allogeneic bone grafts in the defect sites demonstrated the process of involucrum formation (Figure 29).

The root surface of the teeth demonstrated the process of cementogenesis (Figure 47).

2. FRESH TWO-WALLED GRAFT

A. Epithelium

0 Day

Stratified squamous keratinized epithelium demonstrates slight intercellular edema.

3 Day

The epithelium of the 3 day fresh allogeneic bone graft was lost in sectioning.

7 Day

Stratified squamous keratinized epithelium demonstrates slight intercellular edema, acanthosis, and hyperplasia. There is an area of ulceration adjacent to the tooth. An apical migration of the proliferating epithelial cells is seen along the
inner aspect of the flap in contact with the tooth.

14 Day

Stratified squamous parakeratinized epithelium with slight intercellular edema is present. Apical migration of the epithelial cells is present along the tooth surface. Fragments of exfoliating graft tissue are noted.

21 Day

Stratified squamous keratinized epithelium with slight intercellular edema is seen. Some fragments of exfoliating graft tissue are noted. There is an area of thin proliferating epithelium between the flap and the adjacent root surface.

28, 42, and 56 Days (Figure 13)

Stratified squamous parakeratinized epithelium is present with only a slight amount of intercellular edema. The proliferating epithelium continues to surround the exfoliating graft fragments. The epithelium forms a normal attachment to the root surface.

b. Connective Tissue

0 Day (Figure 14)

The full mucoperiosteal flap is detached. The
connective tissue appears to be within normal limits, except for the presence of a slight chronic inflammatory infiltrate which is similar to that seen in a chronic gingivitis. There is blood present between the flap and the tooth extending down to the alveolar crest.

3 Day (Figure 17)

The connective tissue presents moderate vascular dilatation, interstitial edema, and a subacute inflammatory infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and plasma cells. The inflammatory infiltrate is greater adjacent to the graft fragments. There are large areas of amorphous ground substance where the collagen fibers had been lost especially adjacent to the graft fragments. Areas of cellular proliferation, fibroblastic and endothelial, are noted around the blood vessels.

7 Day (Figure 18)

The connective tissue presents vascular dilatation, interstitial edema, and areas of collagen fiber loss apparent where amorphous ground substance is present. A chronic inflammatory infiltrate is noted and it increases in intensity adjacent to the graft
fragments. Fibroblastic and endothelial proliferation is noted emanating from the connective tissue border. Some hemosiderin pigment is seen near the engorged blood vessels of the periodontal ligament space. Many multinucleated giant cells are seen which appear "osteoclast-like."

14 Day (Figure 20)

Dilated capillaries are seen in the connective tissue as well as a slight interstitial edema, and a lessened chronic inflammatory infiltrate. Some areas of amorphous ground substance are present which indicate a loss of collagen fibers. Fibroblastic and endothelial proliferation is noted emanating from the connective tissue border and moving toward the graft material. Hemosiderin pigment is seen adjacent to the engorged blood vessels of the periodontal ligament space.

21 Day (Figure 21)

The connective tissue presents vascular dilatation, increased vascularity, and only slight interstitial edema. There is an increased cellularity due to the fibroblastic proliferation. Multinucleated giant cells are seen in the connective tissue near
some of the graft fragments. The beginning of collagen fiber organization is seen in addition to the areas of decreased collagen degeneration. A mild chronic inflammatory infiltrate is present.

28 Day (Figure 22)

Some dilated capillaries are found in the connective tissue. The collagen fiber bundles are becoming more organized. An increased cellularity is noted due to fibroblastic proliferation. The chronic inflammatory response that is present is typical of chronic gingivitis coupled with the exfoliation of graft fragments.

42 Day (Figure 24)

The flap wound is repaired. There is still an increase in cellularity adjacent to the graft. The collagen fiber bundles are organized. An increased vascularity is still noted. A chronic inflammatory response exceeding that of a typical chronic gingivitis is present due to the presence of an adjacent exfoliating deciduous tooth with the resulting increased inflammation.
56 Day (Figure 27)

The connective tissue shows evidence of repair. The collagen fibers are organized into mature bundles. There is some chronic inflammatory infiltrate present near the epithelium characteristic of chronic gingivitis. Also, there is some edema and an increase in vascularity due to the inflammatory process.

c. Alveolar Bone

0 Day

The alveolar bone adjacent to the defect shows repair from the initial trauma of the defect preparation. The alveolar bone is viable as noted by the presence of osteocytes in the lacunae. There are basophilic reversal lines present which signify recent osteogenic activity. No new bone is seen in the crestal marrow spaces.

3 Day

Osteoblastic activity is seen on the crestal and periosteal side of the alveolar bone. On the periodontal ligament side of the alveolar bone osteoclastic activity is seen instead of osteoblastic activity. All the alveolar bone is viable. Basophilic reversal
lines are present indicating recent osteogenic activity. The periodontal ligament has been replaced by fibrous connective tissue. Osteoblastic activity is noted in the crestal marrow spaces along with a fibrofatty marrow.

**7 Day**

Osteoblastic and osteoclastic remodeling is seen on the alveolar bone. Some osteoid and osteoblasts are seen on the alveolar crest. The crestal marrow spaces also contain osteoblasts and a fibrofatty marrow. The alveolar bone is viable and presents basophilic reversal lines.

**14 Day**

Osteoblastic and osteoclastic remodeling is seen on the alveolar bone. Osteoblasts and osteoid apposition is seen on the crestal, periosteal, and periodontal ligament sides of the alveolar bone. Some of the new crestal bone is seen to mingle with some of the graft fragments. There is a reduction in the size of the crestal marrow spaces due to the deposition of osteoid. Osteoblasts are also seen on the marrow space surfaces. The alveolar bone is viable and basophilic reversal lines are present.
21 Day

Osteoblastic remodeling and bone apposition are seen on the alveolar crest. A few osteoblasts can be seen there associated with the new bone. New bone can also be seen in the crestal marrow spaces. The alveolar bone is viable and basophilic reversal lines are present.

28 Day

Osteoblastic remodeling and bone apposition are seen on the crestal, periosteal, and periodontal ligament sides of the alveolar bone. Also, new bone formation is seen in the crestal marrow spaces. The alveolar bone is viable and basophilic reversal lines are present.

42 Day

Osteoblastic remodeling and bone apposition are seen on the crestal, periosteal, and periodontal ligament side of the alveolar bone. New bone formation is seen in the crestal marrow spaces which accounts for their reduction in size. Fragments of the graft tissue are seen to be incorporated into the newly forming alveolar crest, which can be characterized as an involucrum formation. The involucrum is
non-viable bone while the surrounding of the alveolar bone is viable. There are basophilic reversal lines present.

56 Day (Figures 27, 29, and 30)

Osteoblastic remodeling and bone apposition is seen on the crestal, periosteal, and periodontal ligament side of the alveolar bone. Fragments of graft tissue are seen to be incorporated in the newly forming alveolar crest. During the defect creation, a notch was created in the root surface. The new bone formation follows the root surface into the notch. New bone formation is also seen in the crestal marrow spaces. Basophilic reversal lines are present in the alveolar bone.

d. Fresh Cancellous Bone and Marrow Graft

0 Day Fresh Core (Figures 15 and 16)

The fresh bone core presents a trabecular pattern of cancellous bone which is partially lined with osteoblasts. Between the trabeculae there are areas of fibro-fatty and hematopoietic marrow.
The cancellous bone is viable as evidenced by the osteocytes in the lacunae. Basophilic reversal lines are present. Islands of cartilage and perichondrium are seen where cortical bone and a periosteum is usually seen.

3 Day (Figure 17)

A few of the graft fragments are seen to be exfoliating. All of the graft fragments are non-viable. The fragments are surrounded by a subacute inflammatory infiltrate of polymorphonuclear leukocytes, lymphocytes, and plasma cells. Some areas of fatty marrow can be seen. No osteoclastic or osteoblastic activity can be seen on the graft fragments.

7 Day (Figures 18 and 19)

A few of the graft fragments are seen to be exfoliating. All of the graft fragments are non-viable. The fragments of the graft near the alveolar crest demonstrate osteoblastic and osteoclastic activity. Some osteoid can be seen deposited on these fragments. The fragments are surrounded by a chronic inflammatory infiltrate.
14 Day (Figure 20)

A few of the graft fragments are being exfoliated. The graft is non-viable. Osteoclasts can be seen on the surface of some of the fragments. The crestal fragments have osteoid and bone apposition with osteoblasts on the surface. The fragments are surrounded by a lessened chronic inflammatory infiltrate.

21 Day (Figure 21)

The repairing defect site demonstrates bone graft fragments scattered throughout the young fibrous connective tissue. The graft is still separate from the alveolar crest. The graft fragments are non-viable. A small amount of bone apposition with osteoblasts on the surface of the fragments can be seen.

28 Day (Figures 22 and 23)

The alveolar crest and the graft fragments are now connected by new bone formation from the crest and on the surface of the graft fragments. Some osteoclasts still can be seen on some of the non-viable graft fragments near the alveolar crest. Considerable bone apposition by osteoblasts on the
surface can be seen as some of the graft fragments form an involucrum. Exfoliating fragments of bone are noted.

42 Day (Figures 24, 25, 26)

There are only a few graft fragments that are not attached to the alveolar crest. Osteoblasts and new bone apposition can be seen on the graft fragments. The graft fragments are non-viable but the new bone which forms on the fragments is viable. The beginning of a new periodontal ligament formation can be seen.

56 Day (Figures 27, 28, and 29)

There are still a few graft fragments which are not attached to the alveolar crest. The involucrum formation is continuing as new bone is deposited by osteoblasts on these fragments. The graft bone is non-viable. A new mature periodontal ligament is present which is vascularized.

e. Root Surface and Pulp

0, 3, 7, and 14 Days

If the cementum was not removed during the defect preparation, it is observed to be irregularly
thickened. The dentin remains within normal limits. The pulp tissue varies from normal to chronically inflamed, characterized by vasodilatation, degenerated odontoblasts, and edema.

21 Day

The fibrous connective tissue around the erupting tooth prevented the tooth from coming into contact with the graft, so no effects are seen.

28, 42, and 56 Day (Figure 27)

The cementum appears irregularly thickened. New cementum and cementoid are seen. A layer of cementoblasts line the cementum surface. The dentin appears within normal limits. The pulp tissue shows chronic inflammation. No root resorption is seen.

3. FROZEN TWO-WALLED GRAFT

a. Epithelium

0 Day

Stratified squamous keratinized epithelium demonstrates slight intercellular edema.

3 Day

Stratified squamous keratinized epithelium
demonstrates slight intercellular edema, perinuclear edema, and acanthosis. The epithelium varied in thickness from place to place. Fragments of exfoliating graft are noted.

7 Day

The epithelium of the 7 day frozen allogeneic bone graft was lost in sectioning.

14 Day

Stratified squamous parakeratinized epithelium is seen to demonstrate intercellular edema, acanthosis, and hyperplasia. An apical migration of the epithelial cells is present along the tooth surface. Fragments of the exfoliating graft tissue are noted.

21 Day

Stratified squamous non-keratinized epithelium is seen to demonstrate slight intercellular edema and slight acanthosis. An apical migration of the epithelial cells is noted along the root surface. An epithelial attachment to the tooth is present.

28 Day

Stratified squamous parakeratinized epithelium is present. An apical migration of the epithelial
cells is seen ending in an attachment to the tooth. The epithelium proliferates downward to surround some of the exfoliating graft fragments.

42 and 56 Days (Figures 12 and 13)

Stratified squamous parakeratinized epithelium is present. The epithelium proliferates and surrounds some of the exfoliating graft fragments. The epithelial cells are seen to migrate apically along the tooth surface to the point where they form an attachment to the tooth.

b. Connective Tissue

0 Day

The full mucoperiosteal flap is detached. The connective tissue appears to be within normal limits except that there is a slight chronic inflammatory infiltrate present which is similar to a chronic gingivitis. There is extravasated blood present between the flap and the tooth extending down to the alveolar crest.

3 Day (Figure 33)

Dilated capillaries are seen in the connective tissue interstitial edema, and a subacute inflammatory infiltrate consisting of polymorphonuclear leukocytes,
lymphocytes, and plasma cells. The inflammatory infiltrate is greater adjacent to the graft fragments. There is an increased vascularity with the blood vessels being engorged with red blood cells. Areas of amorphous ground substance are seen where the collagen fibers have been lost. These amorphous areas are found more often adjacent to the graft fragments. There are areas of increased cellular proliferation. The periodontal ligament is replaced by a dense fibrous connective tissue. Also noted are some dislodged fragments of hard tissue which is probably of dental origin.

7 Day (Figure 34)

Vasodilatation, interstitial edema, and a chronic inflammatory infiltrate are seen in the connective tissue. A proliferation of fibroblasts accounts for the increased cellularity. Between the graft fragments there are amorphous areas where the collagen fibers were lost. Endothelial proliferation is noted emanating from the connective tissue border toward the graft tissue. There are many multinucleated giant cells present; those adjacent to the graft tissue appear osteoclast-like. Degenerated red blood cells account for the hemosiderin pigment present.
14 Day (Figure 36)

The connective tissue presents vascular dilatation, interstitial edema, and a minimal chronic inflammatory infiltrate. Areas of collagen loss are apparent where the ground substance appears amorphous. Fibroblastic and endothelial proliferation are seen emanating from the connective tissue border toward the graft fragments.

21 Day

The connective tissue presents slight interstitial edema, vascular dilatation, and a very mild chronic inflammatory infiltrate. The increased vascularity and increased number of fibroblasts are apparent. Multi-nucleated giant cells and hemosiderin pigment are present in the connective tissue and adjacent to the graft fragments. The collagen fibers appear to become organized.

28 Day (Figure 39)

Increased cellularity due to fibroblastic proliferation, increased vascularity, and some multi-nucleated giant cells is descriptive of the appearance of the connective tissue at this time. The amount of chronic inflammation has decreased to the
point where it simulates that of a chronic gingivitis. The collagen fibers are becoming more organized, some collagen bundles are seen.

42 and 56 Day (Figures 41 and 12)

The connective tissue appears to have matured with very organized bundles of collagen fibers. A few exfoliating graft fragments can still be seen. The repair process has not been completed as yet as seen by the presence of an increased vascularity and fibroblastic proliferation. There are a few areas where hemosiderin pigment can be seen in the connective tissue. The inflammation present is similar to that seen in a case of chronic gingivitis.

c. Alveolar Bone

0 Day

The alveolar bone adjacent to the defect shows repair from the initial trauma of the defect preparation. The alveolar bone is viable as noted by the presence of osteocytes in the lacunae. There are basophilic reversal lines present which signify recent osteogenic activity. No new bone is seen in the crestal marrow spaces.
3 Day

Osteoblastic and osteoclastic remodeling is seen on the alveolar bone. Osteoblastic activity is present on the periodontal ligament side of the alveolar bone and in the crestal marrow spaces. These marrow spaces are observed to contain a fibrofatty marrow. The bone of the alveolar crest is observed to be viable and reversal lines are apparent indicating recent osteogenic activity.

7 Day

The alveolar bone appears viable with basophilic reversal lines apparent. The alveolar bone is remodeled by osteoblastic and osteoclastic deposition and resorption. Newly deposited osteoid can be seen on the crest along with some osteoblasts. Osteoblastic activity can be seen in the crestal marrow spaces.

14 Day

Osteoblastic and osteoclastic remodeling is present. Osteoblasts and new bone apposition can be seen on the crestal, periosteal, and periodontal ligament side of the alveolar crest. The new bone apposed over the crest is beginning to mingle with the graft fragments. A reduction in the size of the
crestral marrow spaces is observed due to the osteoid and new bone apposed around the marrow spaces. The alveolar bone is viable and demonstrates basophilic reversal lines.

21 Day

Osteoblastic remodeling is predominant. There are many more osteoblasts than osteoclasts present. The alveolar crest and the graft fragments have almost become one. New bone apposition is observed on the crestal, periosteal, and periodontal ligament side of the alveolar crest. Also, new bone and osteoblasts are seen in the crestal marrow spaces causing a reduction in the marrow space size. The alveolar bone is viable and basophilic reversal lines are present.

28 Day

Osteoblastic remodeling is observed throughout the alveolar crest. New bone apposition and osteoblasts are seen on the crestal, periosteal, and periodontal ligament side of the alveolar crest. Also, new bone apposition is seen in the crestal marrow space further reducing their size. The alveolar crest and the graft fragments have now joined and
become one. Basophilic reversal lines and viable bone characterize the alveolar crest.

42 Day

Further reduction in the diameter of the crestal marrow spaces is seen due to new bone apposition. Osteoblastic remodeling by means of bone apposition is seen on the crestal, periosteal, and periodontal ligament side of the alveolar crest. The graft fragments and alveolar crest having joined together, cause involucrum formation to take place. Non-viable grafts fragments are seen to have viable bone surrounding them. The alveolar crest is viable with the exception of these involucra. Basophilic reversal lines are observed. The presence of an immature new periodontal ligament is noted.

56 Day (Figures 42, 43, and 44)

Osteoblastic remodeling by means of apposition is demonstrated on the crestal, periosteal, and periodontal ligament side of the alveolar crest. Also, there is a further reduction in the size of the crestal marrow spaces due to bone apposition. The alveolar crest and grafts fragments have joined to become one with the resultant involucrum formation.
During the defect creation, a notch was created in the root surface. The new bone formation follows the root surface into the notch. The alveolar crest is viable with the exception of the involucrum formations. Basophilic reversal lines can be seen in the alveolar bone. A new mature periodontal ligament is seen. A silver stain was done to confirm the presence of collagen fibers in the new periodontal ligament. With the silver stain, the periodontal ligament fiber bundles can be seen in the new alveolar bone on one side, progressing through the periodontal ligament space, and inserted into the new cementum opposite the alveolar crest.

d. Frozen Cancellous Bone and Marrow Graft

0 Day Frozen Core (Figures 31 and 32)

The frozen bone core presents a trabecular pattern of cancellous bone which is partially lined with osteoblasts. A fibrofatty marrow, which is partly filled with immature hematopoietic cells, is present between the trabeculae. The cancellous bone is viable as evidenced by the osteocytes in the lacunae. There are some empty lacunae, however, indicating
some non-viable bone. Basophilic reversal lines are also observed. There are islands of cartilage present where cortical bone is usually seen. A periosteum is in evidence.

3 Day (Figure 33)

Some graft fragments are seen to be exfoliating. All of the graft fragments, which are non-viable, are surrounded by a subacute inflammatory infiltrate of polymorphonuclear leukocytes, lymphocytes, and plasma cells. At this point, the graft fragments do not exhibit any osteoblastic or osteoclastic activity. In addition to cancellous bone graft fragments being observed, some bone marrow tissue is also in evidence.

7 Day (Figures 34 and 35)

A few of the graft fragments are seen to be exfoliating. Some of the fragments next to the alveolar crest have some osteoid deposition and osteoblasts present on them. The graft fragments themselves are non-viable. Bone apposition is taking place on non-viable bone fragments. A few osteoclasts are noted in the chronic inflammatory infiltrate.

14 Day (Figures 36 and 37)

A few non-viable graft fragments are being
exfoliated. Osteoclasts are noted around some of the fragments while osteoid and bone apposition by osteoblasts is seen on some of the crestal graft fragments and on a few of the fragments in the middle of the graft. Connective tissue organization is observed to be greater around the graft fragments. The fragments are surrounded by a lessened chronic inflammatory infiltrate.

21 Day (Figure 38)

Graft fragments are seen to have osteoblasts and new bone apposition on the surface. Some osteocytes can be seen incorporated in the lacunae of this new bone. This is the beginning of the involucrum formation. A few of the graft fragments are seen connected to the alveolar crest. A few osteoclasts are present on the surface of some of the non-viable graft fragments, but the osteoblastic activity predominates over the osteoclastic activity.

28 Day (Figures 39 and 40)

Some graft fragments are still observed to be exfoliating. The non-viable grafts fragments have osteoblasts and new bone apposition on the surface. The new bone is viable while the graft fragments that
are being deposited on are non-viable. Very few osteoclasts can be seen at this time. The graft tissue and the alveolar crest have joined and in the process, the non-viable graft fragments have formed involucrums.

42 and 56 Days (Figures 41, 42, 43, and 44)

There are very few graft fragments which are not attached to the alveolar crest. The involucrum formation is continuing as the new bone apposition continues on the surface of these graft fragments. Osteoblasts are plentiful and osteoclasts are rare. A new mature periodontal ligament is present which is vascularized. The fibers of the new periodontal ligament are directed obliquely, going from bone to cementum.

e. Root Surface and Pulp

0, 3, 7, 14, 21 Days

If the cementum was not removed during the defect preparation, then it is observed to be irregularly thickened. The dentin remains within normal limits. The pulp tissue varies from normal to chronically inflamed, characterized by fibrosis,
edema, vasodilatation, and degenerated odontoblasts.

28, 42, and 56 Day (Figures 39, 43, and 44)

The cementum appears irregularly thickened. New cementum and cementoid are seen. A layer of cementoblasts line the cementum surface. No root resorption was seen in the frozen grafts. The dentin appears within normal limits. The pulp tissue also appears to be within normal limits.

4. TWO-WALLED CONTROLS

a. Epithelium

7, 21, 42 Day

The epithelium demonstrates similar findings to those observed in the fresh and frozen bone allografts. Intercellular edema, slight acanthosis, and some atrophy characterize the stratified squamous keratinized epithelium. A new epithelial attachment is formed to the root surface at a more apical level.

b. Connective Tissue

7, 21, 42 Day (Figures 45 and 46)

The connective tissue is also similar to that in fresh and frozen allografts as well as in the control
defects. It presents vascular dilatation, interstitial edema, and a chronic inflammatory infiltrate which gradually lessens. The collagen fibers change from areas of large fiber loss and amorphous ground substance, to an increased collagen fiber organization, and finally, to very organized, mature collagen bundles. Fibroblastic and endothelial proliferation is noted.

c. Alveolar Bone

7 Day

The alveolar bone is viable throughout and basophilic reversal lines are noted. Some osteoid and new bone can be seen deposited on the alveolar crest on the crestal, periosteal, and periodontal ligament sides. The bone apposition at this point is probably the repair response to the initial defect preparation.

21 Day

The alveolar bone is viable throughout. Basophilic reversal lines are seen in the old alveolar bone. New bone trabeculae are observed extending from the old alveolar bone crest into the defect. Osteoblasts are present surrounding the new bone.
New bone apposition is also noted on the crestal, periosteal, and periodontal ligament side of the alveolar crest. No basophilic reversal lines are seen in this newly deposited bone. The new bone is viable as seen by the osteocytes present in the lacunae.

42 Day

Osteoblastic remodeling by means of new bone apposition is seen on the crestal and periosteal sides of the alveolar bone. There is a reduction in the size of the crestal marrow spaces due to osteoblastic activity. The alveolar bone is viable and basophilic reversal lines are seen in the older alveolar bone but not in the newly deposited bone.

d. Defect Site

7, 21, 42 Day (Figures 45 and 46)

Initially, the fibroblasts and endothelial cells are seen to proliferate from the adjacent bone marrow spaces and from the connective tissue of the inside layer of the mucoperiosteal flap which was reapposed following the curettage of the defect. The tissue filling the defect becomes vascularized and the connective tissue matures. New bone apposition is seen from the alveolar crest into the defect.
e. Root Surface and Pulp

7 Day

The cementum demonstrates an irregularly thickened appearance, similar to that seen in the fresh and frozen allografts. The dentin is seen to be within normal limits. The pulp tissue appears chronically inflamed as evidenced by edema, hemorrhage, lymphocytes and plasma cells, and the absence of odontoblasts.

21 Day (Figure 47)

The cementum appears irregularly thickened. Also, new cementum and cementoid are seen. A layer of cementoblasts line the cementum surface. Some reparative dentin is noted. The pulp is chronically inflamed and demonstrates fibrosis, edema, and an absence of odontoblasts.

42 Day

The section of the tooth surface adjacent to the defect was lost in processing. The pulp tissue demonstrates chronic inflammation.
CHAPTER V

DISCUSSION

The results of this study are promising in that osseous regeneration and reattachment were obtained in the two-walled osseous defects by using both fresh and frozen allografts. Even though surgical curettage did produce osseous repair, it was delayed and not to the same degree, compared to that elicited by the graft. In addition, the sequential healing process was the same for both the fresh and the frozen allografts. The osteogenic potential of both allografts was also similar.

The frozen allograft did evoke a less intense inflammatory response when compared to that of the fresh allograft. The difference could possibly be due to the preservation process which did decrease the viability of the frozen graft. If the viable osteocytes in the bone are the source of antigens causing the foreign body reaction as reported by Heslop\textsuperscript{1C3} and Billingham,\textsuperscript{107} then the freezing process could have decreased the amount of antigenicity by decreasing the number of viable cells. It is also possible that the frozen allograft caused a delayed vascular invasion of the graft which also could account for this less intense inflammatory response as reported by Bonofiglio et al.\textsuperscript{97}
ROOT RESORPTION

Root resorption in the area of the transplant did not take place when either fresh or frozen allografts are used as the present study has shown. This phenomenon has been a problem that has been observed by several researchers who have utilized fresh iliac autografts.\textsuperscript{4,6,58,110,117-120} A possible explanation of the root resorption seen in fresh autogenous hip marrow grafts is that the bone forms so fast that the cementum does not have time to form as suggested by Siebert.\textsuperscript{120} In the case of the allograft, the immune response may diminish the rate of new bone formation and thereby allow sufficient time for cementogenesis to occur. Cementogenesis began at day 21 in the fresh allografts, frozen allografts, and the control sites. It does not appear that the allografts, either fresh or frozen, delay or suppress cementogenesis as was claimed by Morris.\textsuperscript{121-123}

EXPERIMENTAL DEFECTS

The experimental defects created in this study simulated chronic periodontitis both clinically and histologically. This ability to create reproducible chronic osseous defects affords a needed control in this experiment. Although the introduction of the toothpick irritant into the bony defect allowed the ingress
of bacteria and their by-products into the defect causing chronic inflammation, the created defects are not identical to those found in the naturally occurring periodontal disease process. Since all of the etiologic factors of periodontal disease are not known, we can possibly produce only some of them in the experimental animals. The etiologic factors affect not only the disease process but also the repair process. The possibility exists that the healing process is affected by the absence of some unknown etiologic factors.

**EPITHELIUM AND CONNECTIVE TISSUE REPAIR**

The gingival epithelium demonstrated swift and extensive clinical and histological regeneration and repair in our study. The replacement of destroyed tissue requires mobilization of cells from the margin of the wound, and their centripetal migration into the surgical wound; this is the first step in restoring continuity of epithelium. Two distinct processes are seen in the mobilization of cells: 1) there must be a sufficient number of cells available and these are supplied by local mitotic division, and 2) there must be a movement of epithelial cells into the wound area. The stimulus for this epithelial migration appears to be the lack of contact of the marginal epithelial cells of the flap with each other or with the tooth surface. As the migrating
cells move beneath the clot, they digest fibrin and damaged extracellular substances in the process. It has been proposed that epithelial cells produce collagenase, fibrinolysin, and other related proteolytic enzymes, which makes the pathway of migrating cells possible. As the wound begins to repair, fragments of sequestrated bone are often seen being enclosed in reparative epithelium ("epithelial forceps"), which appear to facilitate their eventual extrusion.

The connective tissue, like epithelium, regenerated and repaired rapidly. Fibroblasts and endothelial cells soon become the dominant cells of the wound. The restoration of the continuity of the connective tissue involves four processes: 1) fibroblastic proliferation, 2) migration of new fibroblasts into the wound area, 3) formation of new extracellular substance, and 4) remodeling of the extracellular material in the wound.

Grillo found it impossible to separate the proliferative response of the capillaries from that of the adjacent fibroblasts. This fibroblast-capillary system appears to represent a primary reparative response. The fibroplasia is accompanied by and dependent upon an intimate blood supply. It is the blood supply which transports nutrients necessary for mitosis, in addition to those supplied locally, to the proliferating fibroblasts. In the early stages of healing, it is necessary that new blood vessels supply the deficient wound area. These are provided by mitotic
division of the endothelial cells of preexisting capillaries which produce new sprouts. The endothelial cells of the pre-existing capillaries retain their blastic potential and reproduce the parent structure by division, budding, migration, and differentiation. New vessels migrate into the wound area along with the fibroblasts. There appears to be a relationship between the roles of epithelium and connective tissue in the wound healing process since the connective tissue response (fibroblastic and endothelial proliferation) is stimulated by epithelial proliferation and, later on, the new young connective tissue inhibits the proliferation of the epithelium.\textsuperscript{127,131} It was observed in this study, as well as in others,\textsuperscript{128,132} that the onset of fibrinogenesis occurs after the migration of the epithelial cells has been initiated, possibly, by some positive feedback mechanism as yet unknown. In light of these facts, it might be best not to overfill the defect with the graft material because primary closure of the flap is important. Any excess bone that is exposed to the oral environment will undergo necrosis and thereby delay the wound healing process. This delay will result in prolonged acute inflammation, and the delayed vascularization of the graft area and delayed organization of the connective tissue essential for new bone formation. Each will cause a decrease in the amount of osseous regeneration in the defect.
Bone repair and regeneration was found to be similar in both the fresh and the frozen allografts. Necrosis occurred early after insertion of the graft probably due to the lack of a blood supply. This necrosis was followed by osseous regeneration. There are two types of connective tissue cells which can produce new bone. The first type, osteogenic cell, has an inherent potential for producing bone when stimulated by the "bone induction principle." The natural potential of the second type of cell is to produce connective tissue, but it will become an osteogenic cell if induced to do so by an osteogenic stimulus -- the "bone induction principle." The cells possessing inherent osteogenic potential for bone production are found in the endosteum, haversian systems, bone marrow, and the cambium layer of the periosteum of young bones. The perivascular undifferentiated mesenchymal cells are drawn into osteogenesis and are transformed into osteoblasts under the influence of the "inductive mechanism" as reported by Trueta. When the periodontal osseous defect is considered, a number of sources come to mind from which perivascular cells can originate: 1) the periosteum, 2) the haversian system, 3) the connective tissue above the defect, 4) the capillaries of the periodontal ligament space, and 5) the marrow spaces of the alveolar crest. Since the alveolar crest was the first place to show any bone formation, it appears that the
marrow spaces are the dominant source of perivascular undifferentiated mesenchymal cells. Furthermore, it has been demonstrated by Melcher and Irving\textsuperscript{135} in similar types of osseous wounds that endosteal cells and marrow are responsible for most of the osteogenesis.

Available evidence seems to implicate the undifferentiated mesenchymal cell as the precursor of osteoclasts.\textsuperscript{136} A recent electron microscopic investigation claims that osteoblasts and osteoclasts are each derived from different, morphologically distinct, perivascular precursor cells.\textsuperscript{137} It seems more plausible that the various types of cells found in and around bone are different functional states of the same cell. This would mean that the osteoclasts disassociate into apparently less specialized cells (osteoprogenitor cells) which are then available once again for differentiation into either osteoblasts or osteoclasts.\textsuperscript{138-140} Likewise, osteoblasts can "dedifferentiate" into less specialized cells, the undifferentiated mesenchymal cells, and can then, if needed, differentiate into osteoclasts as suggested by Bloom et al.,\textsuperscript{138} Tonna,\textsuperscript{139} and Young.\textsuperscript{140}

**INDUCTION**

The regeneration of new bone seen following the grafting procedure demonstrated the inductive power of the allograft
fragments. The "inducing substances" possibly react with receptor sites on the cell membranes of the responding cells, causing a message to be sent to the genetic material in the nucleus of that cell to differentiate into a bone producing cell. Whether the "inducing substance" is a pattern or template of surface macromolecules, or a diffusible small protein that acts as the substrate for an enzyme are matters of speculation, but the intracellular reactions that follow may develop from feedback reactions from either cytoplasm to gene, or double cycle reactions from cytoplasm to cytoplasm as reported by Waddington. The graft fragments may contain the "inducing substances" and therefore add to the "inducing substances" already released as a result of the curettage of the defect. This could account for the difference in the degree of new bone formation seen in the grafted defects when compared to the curetted control defects. As the graft became necrotic, it may have released the "inducing substances." In fact, cell death itself is probably an inducer of osteogenesis because it releases "inducing substances." Urst postulated that the bone induction principle is:

1) one or more acid-insoluble proteins of macromolecular dimensions, 2) associated with but not inseparable from the antigenic activity, 3) transmitted short distances but not free in solution in extracellular spaces, 4) destroyed by amounts of heat, cryolysis, radiation, and cytoplasmic poisons that denature
proteins, and 5) an integral part of the cell slime and condensed glycoproteins of the cementing substances. The nature of this stimulus is of great interest, not only academically, but also clinically, because it is the key to the control of bone formation. As yet, however, no substance has been isolated which fulfills all the properties of the bone induction. These "inducer substances" probably triggered cells of the defect to differentiate into osteoblasts. The "bone induction principle" is also present in the organic matrix of bone tissue free of the osteocytes. Because only small amounts of "inducing material" may be needed to induce osteogenesis, care should be taken in attributing the origin of the "bone induction principle" to any single component of the tissue.

The immune response to the allograft may slow down the induction system. Although compared to a recent study utilizing iliac autografts, the repair rates appeared similar. Since the frozen allograft appeared to be morphologically less viable due to the freezing process, it is possibly less antigenic also. This could be the reason why the frozen allografts appeared to be slightly ahead of the fresh allograft in the healing process. The fact that the allografts used in this study are small quantitatively when compared to those used in the field of orthopedics could account for the slight lag in healing between the fresh and frozen bone grafts. With larger allografts possibly a greater
difference would exist between the healing rates of the fresh and frozen grafts.

A higher osteoclast concentration appeared to precede an increase in the osteoblastic concentration. It is possible that the osteoclastic resorption is also capable of acting as an inducer of undifferentiated mesenchymal cells to differentiate into osteoblasts. There are two possible means by which osteoclastic resorption can act as an inducer. First, the osteoclastic resorptive process may release an "inducer substance" by the resorption of mature bone. Secondly, some of the secretory material released from the osteoclasts may act as an "inducer," in addition to its primary function of dissolution of the organic matrix of bone.

Some non-vital graft fragments were found incorporated as involucrum in the regenerating alveolar crests. Others were exfoliated (sequestra). These sequestrations could be due to the overpacking of the osseous defect with graft material or to the immunologic rejection phenomenon to the allograft. It probably is due to the overpacking of the defect because it was also observed in a recent study using iliac autografts.6

New bone can be seen deposited on the surface of the graft fragments, eventually becoming incorporated into the new network of cancellous bone. The graft appears to act as a matrix upon which new bone is laid down. The graft is slowly resorbed and
replaced with new bone. This resorption occurs on the outer surface of the graft and on the inner surface of the haversian canals of the graft. The ultimate fate of the grafted bone is resorption and replacement, but this will take considerable time. As the graft resorbs, it releases calcium and other minerals into the area. These minerals, along with those supplied by the circulation, could then probably be utilized in the calcification of the new bone being deposited.

IMMUNE RESPONSE

The factor of greatest interest in tissue grafting, both experimentally and clinically, is the immune response of the host to the allograft. It should be kept in mind that it is not appropriate to make exact comparisons between bone grafts and grafts of other tissues, such as skin or kidney. In the skin or kidney grafts, the host is expected to accept the living tissue, nourish it, and maintain it without replacement. However, in the bone grafts, incorporation and replacement of the grafts by the host is useful as the quicker the surrounding tissue can be induced to form new host bone, the better. In fact, the survival of the allogeneic donor cells might even lessen the probability of acceptance.
Both the fresh and the frozen allografts underwent necrosis early in this study probably due to the lack of vascular supply. Some of the necrotic bone is used as a matrix upon which new host bone can be deposited resulting in the involucrum formation. Since none of the allogeneic bone is seen to proliferate, we can assume that when necrosis of the allografts occur, the immune response becomes less of a factor. This could be a reason why the frozen allograft may have a head start in that it is possibly less viable at the start.

The allogeneic bone grafts lead to an inflammatory reaction which is best characterized as a delayed reaction that is fundamentally cellular as opposed to humoral in nature according to McLean and Urist. Research by Billingham indicates that when the viable allogeneic bone tissue is transferred, antigens pass directly via the host lymphatics to the regional lymph nodes of the host where the antibodies are formed; the spleen also reacts to the antigens reaching it through the blood, but this response is quantitatively of lesser importance. The antigens in allogeneic bone lead to the production of antibodies by lymphocytes which are mainly produced in the regional lymph nodes and released into the circulation. As long as the transferred allogeneic bone proliferates or remains viable, it continues to form antigens and elicit an immune response from the host.
CONTROL DEFECTS

Osseous regeneration did occur in the control defects at a slower rate and in a different pattern of apposition. The osteoblastic activity was seen only on the bony walls of the defect and on the base of the defect. None was seen originating from the organizing connective tissue of the defect itself. This may explain why the control defects repair at a slower rate and lends credence to the osteogenic potential of the allografts.

CLINICAL APPLICATION

The addition of the allogeneic bone transplant to the list of surgical procedures available to the periodontist definitely enhances the ease with which treatment can be delivered. The necessity of utilizing bone from the patient's hip for grafting intraorally is eliminated. Hopefully, a bone bank could be made available to periodontists, from which they could order the necessary bone in the desired quantities. In the case of a severe periodontal defect, where osseous recontouring would remove too much supporting bone, extraction of the tooth would necessitate extensive and expensive restorative dentistry; the utilization of an allogeneic bone graft in the defect would be a convenient alternative.
Since the frozen iliac crest transplant was histologically and clinically as successful as the fresh, the concept of banking bone appears very plausible. In fact, the frozen bone was possibly more acceptable immunologically due to its probable decreased viability. The long term prognosis of allogeneic transplants cannot be ascertained from this study since the follow up extended only through a two month period. Nothing appeared during the two months though, which would imply that the procedure was anything but very promising. Further research is needed in this area to establish the long term prognosis. It is important to keep in mind that this was an animal study and that further studies using human material are necessary to give satisfactory insight into the validity of this procedure.
CHAPTER VI

CONCLUSIONS

1. Clinically and histologically there was no significant difference in the sequential healing of the fresh and frozen allogeneic iliac crest transplants.

2. Allogeneic iliac crest transplants induce the formation of a new attachment apparatus in primates consisting of new bone, new cementum, and a new functionally oriented periodontal ligament.

3. Both the fresh and the frozen allogeneic iliac crest transplants were accepted by the host as was evidenced by the involucrum formations.

4. Both the fresh and the frozen allogeneic iliac crest transplants induced osseous regeneration more rapidly when compared to surgical curettage. This early bone formation with the resulting decrease in apical migration of the epithelial attachment into the two-walled chronic periodontal osseous defect lessens the probability of pocket reformation.

5. The experimental defects utilized in this study simulate chronic periodontitis and are useful in studying the healing phenomenon.
CHAPTER VII

SUMMARY

A study was conducted to examine histologically the sequential healing phenomena in chronic two-walled periodontal osseous defects following their correction with fresh and frozen allogeneic iliac transplants.

Four adult rhesus monkeys were utilized as experimental models providing 19 specimens from 0 to 56 days postoperatively. There were sixteen graft specimens, and three that served as control specimens in which the osseous defects were treated by surgical curettage only.

The fresh and frozen graft materials were obtained and utilized in the following manner. Seven days prior to defect correction with the graft, cores of cancellous bone and marrow were obtained from the iliac crest of one of the monkeys other than the one that was to receive the graft. The graft materials were glycerolized and subjected to controlled freezing to \(-197^\circ C\). At the time of defect correction, the frozen graft materials were thawed, deglycerolized, and implanted into the osseous defects. At this time, fresh cores of cancellous bone and marrow, obtained
from a different monkey than the one receiving the graft, were also implanted into the osseous defects.

Histologic sections from each specimen were stained with hematoxylin and eosin, a description of the sequential healing events was recorded, and the similarities and differences in repair were discussed.

No significant difference was observed in the sequential healing of the fresh and frozen allogeneic bone grafts, and both fresh and frozen allografts induced a more rapid osseous regeneration than the controls.

The fresh and frozen allogeneic transplants were accepted by the host as was evidenced by the involucrum formations.
<table>
<thead>
<tr>
<th>Old</th>
<th>New</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Autograft</td>
<td>Autograft</td>
<td>same individual</td>
</tr>
<tr>
<td>Isograft</td>
<td>Synograft</td>
<td>identical twins</td>
</tr>
<tr>
<td>Homograft</td>
<td>Allograft</td>
<td>within same species</td>
</tr>
<tr>
<td>Heterograft</td>
<td>Xenograft</td>
<td>different species</td>
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<tr>
<td></td>
<td>Alloplast</td>
<td>bone substitute</td>
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CHAPTER VIII

ILLUSTRATIONS
Figure 1. Preoperative appearance of the experimental area. Note the slight marginal and papillary gingivitis.
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Figure 2. Skin over hip 7 days after cores were removed. Sutures in place prior to their removal.
Figure 10. Skin over hip 7 days after cores were removed. Sutures were removed prior to picture.
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REFERENCES


APPROVAL SHEET

This thesis, submitted by Robert C. Poulsom, has been read and approved by three members of the faculty of the Department of Oral Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

5-6-75

Signature of Advisor