Histological analysis of Bovine Bone grafting using the rat tibia model

by

Pierre Monaghan

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Faculty of Dentistry
Mc Gill University
Montreal, Quebec, Canada

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Name PIERRE MONAGHAN

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ABSTRACT

Experimental investigations were carried out concerning the use of bovine bone (Unilab Surgibone) grafted in the tibiae of rats. The first experiment evaluated tissue response of bovine bone as an inlay graft and the second experiment as an onlay graft. Histological and morphometric analyses were performed in order to obtain baseline data on tissue response for future experiments using titanium implants with bovine bone grafts in this model. Light microscopy demonstrated rapid incorporation of the inlay graft by new bone, whereas, the onlay graft was mainly encapsulated by fibrous tissue. However, a residual increase in the thickness of the outer cortex of the tibiae was observed with onlay graft. From the results of this study it appeared that Unilab Surgibone was biocompatible and did not induce a foreign body reaction. Future investigations using titanium implants in combination with the bovine bone grafts appears to be possible especially if an inlay \ onlay design is attempted.

RÉSUMÉ

Nous avons procédé à des expériences de greffes d'os bovin (Unilab Surgibone) au niveau du tibia du rat. La première partie s'intéressait à une greffe d'os bovin à l'intérieur du tibia soit dans la cavité médullaire. La seconde partie consistait à greffer l'os bovin sur la surface externe du tibia. Des analyses histologiques et morphométriques ont été réalisées afin d'obtenir des données de base en vue d'une future expérience qui combinerait des implants en titanium avec l'os bovin chez le rat. L'examen au microscope optique a démontré une incorporation osseuse rapide des particules d'os bovin dans la cavité médullaire du tibia. Par contre, lorsque utilisé sur la surface externe du tibia, l'os bovin fut encapsulé par un tissu fibreux dans sa majeure partie. Toutefois, la partie inférieure de la greffe fut incorporée par de l'os nouveau résultant ainsi à une augmentation de l'épaisseur de la surface externe du tibia. Il apparaît à la lumière de ses résultats que l'os bovin est biocompatible et ne semble pas induire de réaction à corps étranger chez le rat. Nous prévoyons utiliser dans un avenir rapproché, des implants en titanium de concert avec une greffe d'os bovin en utilisant un modèle de greffe intra et extra médullaire.

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INTRODUCTION

Titanium implants are commonly used in prosthetic dentistry to replace missing teeth (Branemark, 1985). The basic principle behind implants is that posts made of titanium are surgically inserted into human bones, thus providing the foundation upon which a prosthetic device, such as a denture, can be attached.

The term "osseointegration" coined by Professor P.I. Branemark of Sweden, describes the end result of a process of bone growth at the interface between titanium and bone (Branemark.1985). The titanium framework must become completely incorporated in the living bone, and the mineralized tissue completely congruent with the micro-irregularities of the titanium surface. The nature of the tissue at the interface will determine the success or failure of the implanted material (Branemark,1983).

The surgical procedure which provides for osseointegration has well documented clinical success rates of 92% to 98% (Albrektsson,1988). However, this can only be achieved if the quality and quantity of bone at the implanted site is adequate (Leckholm,1985). Following tooth loss there is an associated loss of alveolar bone, such that the remaining bone may be insufficient for dental implantation. For these patients titanium implants may be placed in combination with various types of grafting materials (Garey, 1991). The titanium implants can be installed immediately with the graft material or later after thc graft has healed.

Traditionally autogenous bone grafting has been used widely for reconstructive procedures in the human jaws (Marx, 1983) and more recently in combination with dental implants (Hall, 1991; Collins, 1991). In many instances, the operation for harvesting bone may be more invasive than the placement of

the dental implant. The procurement of autogenous bone is associated with some risks and usually involves hospitalization for several days (Younger, 1989). The search for an ideal grafting material, that could replace autologous bone grafting, is one of the most challenging problems in implant dentistry.

The literature exploring solutions to this problem, including various grafting materials, and mechanisms of healing, will be presented. More specifically, this review will focus on the evolution of bovine bone as an alternative. This grafting material has the advantage of being readily available. Furthermore, it does not require an additional surgical procedure for harvesting. Bovine bone was used in the 1960's and 70's and then it was gradually abandonned in the 1980's. Bovine bone products of the 90's have shown improvement in quality and processing, and consequently, this has resulted in renewed interest in the surgical community.

Before reviewing the different types of bone grafts available, problems associated with edentulism in the maxilla and mandible and the placement of dental implants will be examined. Following this, a review of the general principles of bone graft healing will be presented. These principles were based on autologous bone grafting and may be useful for comparison when reviewing other grafting materials.

This study will explore the potential of bovine bone (Unilab Surgibone) to increase bone volume when used as an inlay and an onlay graft. It is expected that these investigations will provide some insight as to Surgibone's ability to act as a solid base for future dental implant placement. The experiments carried out for this thesis will provide baseline histological data of the bovine bone grafts (Unilab Surgibone) placed in and on the tibiae of rats. It is hoped that results of these investigations will serve as a

template for future studies which will:

1. examine whether a titanium implant can successfully osseointegrate in the bovine bone graft material.

2. determine the ideal time for titanium implant placement in the rat model following bovine bone grafting.

3. determine whether the bovine bone graft will affect the quality of the bone next to the metal implant.

THE PROBLEM; THE EDENTULOUS JAW

The alveolar process is formed in response to the developing and erupting teeth. Once the teeth are lost, the remaining alveolar bone undergoes resorption, remodeling and recontouring (Tallgren, 1972). Furthermore, longitudinal studies indicate that residual alveolar bone appears to be vulnerable to denture wearing and responds by an ongoing and insidious reduction that is irresversible and probably inevitable (Tallgren, 1972). It has also been suggested that hormonal imbalances may be an important factor particularly in females (Nordin, 1975).

Post-extraction alveolar ridge resorption combined with maxillary sinus pneumatization often present limited bone for endosseous implant placement in the posterior maxilla. The maxillary sinus floor is often within a few millimeters of the crest of the edentulous ridge and thus will not support endosseous implant.

The restoration of maxillary posterior edentulous spaces without a posterior abutment has complicated implant protocols for many years. One answer is grafting the maxillary sinus to encourage new bone formation in the pneumatized area prior to placement of a dental implant (Hall, 1991). If the maxillary and the mandibular ridges are also atrophic, the standard surgical treatment is onlay augmentation with autogenous iliac crest bone (Adell, 1990; Boyne, 1985).

A technique for bone grafting of the maxillary sinus via a transalveolar approach was first described by Tatum in the late 70's (Tatum, 1977). More recently, Boyne & James described a technique of maxillary sinus grafting via a horizontal vestibular incision and lateral maxillary ostectomy (Boyne, 1980). Today, the sinus lift technique is the most common approach to the problem (Smiler, 1992) and the standard

graft material is autologous bone harvested from the iliac crest (Raghoebar, 1993). To our knowledge, no longterm prospective studies have been conducted examining sinus lift procedures combined with allogenic or xenogenic grafts. Only a few clinical trials have been reported (Smiler, 1992; Garey, 1991).

The alveolar process of the mandible is also subjected to resorption following extraction of teeth. This process is similar to that of the previous discussion. In the severely atrophic mandible, the bone has become so resorbed that the mental nerve lies on the crest of the residual ridge. When endosteal implants are required in the posterior aspect of the mandible, the height of bone in relation to the inferior alveolar canal becomes critical. In this region, the risk of damaging the inferior alveolar nerve frequently becomes a contraindication for this treatment modality. Therefore, these patients have no choice but to undergo grafting procedures prior to the insertion of dental implants.

Historically, several surgical techniques and grafting materials have been used to augment the vertical height of the mandible in order to improve denture stability (Sugar, 1982; Harle, 1975; Frost, 1982). It is difficult to find many studies which deal with the placement of an endosseous implant (dental implant) associated with an augmented mandibular alveolar ridge. As with traditional preprosthetic surgery, the approach consisted of transplanting autologous bone over the existing residual bone. In the case of extreme mandibular atrophy, grafts for immediate implants may be onlayed over the alveolar ridge in inlay saddle, veneer, and split mandibular configurations (Collins, 1991). However, only preliminary documentation of the combined use of dental implants and transplants (autologous) exists, and the long term outcome of such treatment is not known (Kahnberg, 1989; Keller, 1987).

HEALING OF BONE GRAFTS

Two basic methods are used for accomplishing bone reconstruction: transplantation or implantation. Transplantation involves the surgical transfer of living tissues or organ systems. The cells of the transplant must survive the grafting procedure. Implantation, by contrast, involves surgical transfer of non-living tissues that may be clinically successful even though the cells of the graft do not survive the surgical procedure.

1. Types of bone grafts

Several types of bone grafts can be used in reconstruction surgery. A useful classification groups the bone grafts according to their origin (Bernard, 1991).

a. Autogenous grafts

Autogenous grafts, also known as autografts or autologous grafts, are composed of tissues procured from the same patient. In other words, another body site in the same individual is used as the graft source. Autogenous bone grafts can be obtained from a host of body sites and can be taken in three different forms: corticocancellous, cancellous and cortical.

The iliac crest is the most common source of these grafts, other common sites are ribs, tibial tubercule, and parietal bone. Cancellous bone grafts consist of medullary bone and the associated endosteum and hematopoietic marrow. Due to their particulate nature, cancellous bone grafts produce the greatest concentration of osteogenic cells.

b. Homologous grafts

Also known as allografts or allogeneic grafts, are taken from another individual within the same species. Because the individuals are usually genetically dissimilar, the graft must be treated by various methods to reduce antigenicity. The most common method is lyophilization (freeze drying).

c. Heterogenous grafts

Heterogenous grafts, also known as xenogenic grafts or heterografts, are taken from one species and transfered to another. The use of bovine bone in human, for example, constitutes a xenogenic graft. The antigenic potential of these grafts is much greater than of homologous bone. As we will discuss, heterogenous grafts must be scupulously treated to prevent rejection.

d. Alloplastic grafts

A graft of inert synthetic materials, which is sometimes called implant material rather than graft material (ceramics, plastics, hydroxyapatite, etc...).

2. General principles of bone graft healing

In order to understand the complexity of the healing mechanism of different graft materials, it is important to review the general principles of autogenous bone graft repair. When bone is transplanted from one area of the body to another, several processes are initiated during graft incorporation. Surgeons manipulate bone and tissues to augment bone volume utilizing one or a combination of three mechanisms; osteogenesis, osteoconduction, and osteoinduction (Burchardt, 1983).

Osteogenesis is defined as the formation of new bone from osteoprogenitor cells (mesenchymal cells) (Urist, 1976). There are essentially two types of osteogenesis which can be manipulated.

Spontaneous osteogenesis: which is the formation of new bone arising from osteoprogenitor cells within the wound defect, and

Transplanted osteogenesis: which is the formation of new bone arising from osteoprogenitor cells placed into the wound defect from a distant site.

It is important to mention that osteoprogenitor cells of bone marrow are residual stem cell precursors which can differentiate into any one of the connective tissue lineage's of bone, cartilage, fibrous tissue, muscle, fat, and even blood cells (Caplan, 1991). Furthermore the population of these cells continually diminishes with age, making bone less regenerative and more osteoporotic with time. The ratio of stem cell precursors in human marrow is 1 per 10,000 at birth, diminishes by a factor of 10 to 1 per 100,000 at the teenage years and continues to diminish to a ratio of 1 per 2,000,000 by the age of 80 years (Haynesworth, 1992). The importance of this regression is that no matter what the bone grafting technique, the outcome and maintenance of the graft will be influenced and diminished by this age related factor.

Gray (1979) demonstrated that osteogenesis in cancellous grafts proceeded 2,2 times faster than in cortical grafts of the same weight and 1,7 times faster than in cortical grafts of the same volume. In both types of grafts, approximately 60% of osteogenesis arise from endosteal cells and marrow stroma and 30% from periosteal cells (Gray, 1982). Interestingly, the free, hemopoietic, marrow cells made no contribution to osteogenesis. Thus the composition of the bone graft greatly influences its potential for osteogenesis.

Osteoconduction is defined as the formation of new bone from host derived or transplanted osteoprogenitor cells along a biologic or alloplastic framework (Burchardt, 1983).

This mechanism is exemplified by the bone formation in a tooth extraction socket where bone cells from the lamina dura and periodontal ligament space migrate over the fibrin clot to regenerate bone within the space. It is a phenomenon also observed along reconstruction plates and along frameworks of hydroxyapatite (Bernard, 1991). The process of capillary ingrowth, perivascular tissue, and osteoprogenitor cells from the recipient bed into the graft is also called osteoconduction.

In viable bone grafts, osteoconduction is facilitated by *osteoinduction* processes and therefore occurs more rapidly than in nonviable nonbiologic materials (Urist, 1972).

Osteoinduction is the process in which mesenchymal cells from the recipient bed in contact with the grafted bone matrix will differentiate into bone forming cells (Huggins, 1970; Urist, 1976; Landesman, 1986). Bone induction can also be defined as the mechanism where by a tissue is influenced to form osteogenic elements. Induction requires an inducing stimulus, e.g. a piece of bone or an osteogenic cell, and an environment favorable for osteogenesis (Burwell, 1965; Urist, 1967; Urist, 1976).

The process is thought to be regulated by insoluble polypeptide proteins (growth factors such as bone morphogenetic protein or BMP), specific enzymes, and enzyme inhibitors (Urist,1974; Urist, 1975). The original bone inductive protein discovered by Marshall Urist, in 1965, was termed bone morphogenetic protein (BMP) which was later successfully isolated from decalcified bone matrix of human and bovine tissues (Urist, 1984). Insertion of BMP in thigh

muscle pouches of mice or bone defects of animals and humans consistently induced generation of new bone (Nilsson, 1985; Lovell, 1989). Eight BMP's have been identified and it is believed there are others yet to be discovered. BMP-2 and BMP-7 have already been sequenced and are being synthetized using recombinant DNA technology (Wang, 1990). Further clinical applications of recombinant human bone morphogenetic proteins are also expected in the near future (Wang, 1990).

In nature, BMP's are a part of the transforming growth factor-Beta (TGF-B) super family of connective growth factors and are found in large concentrations in cortical bone (Urist, 1971). It's role in the adult skeleton seems to be that of coupling bone resorption to new bone apposition. When an osteoclast resorbs mineral matrix it releases BMP which, in turn, will recruit and differentiate stem cell percursors to form new bone. In other words, for the normal remodeling of bone, BMP is involved in adaptation to environmental changes, repair of micro-damage to bone, and in bone healing (Wang, 1988).

3. The two-phase theory of osteogenesis

According to this theory, two basic processes occur when bone is transplanted from one area to another within the same individual (Axhausen, 1956, Burwell, 1964; Gray, 1979; Gray 1982). The first phase which is referred to as the cellular phase begins when transplanted cells within the graft proliferate and form new osteoid. This occurs within the first few weeks following transplantation (Burchardt, 1983).

The cancellous cellular marrow contains endosteal osteoblasts and marrow stem cells which will survive the initial harvest-transplant cycle and live via a plasmatic circulation of diffusable oxygen and nutrients prior to

revascularization from the host (Deleu, 1965; Gray, 1982). A prexisting rich capillary density enhances such diffusion during the early critical days before revascularization takes place.

Osteocytes in mineralized bone are less likely to survive transplantation due to their state of differentiation and to their tenuous nutrient supply via haversian vessels and canaliculi (Axhausen, 1956). However, another study showed that osteocytes appear to survive transplantation if they are less than 0,3 mm from the surface (Heslop, 1960).

This first phase is also characterized by the ingrowth of new blood vessels around the graft, which bring nutrients to the transplanted cells (Arora, 1964; Deleu, 1965). Evidence also exists suggesting that cancellous bone is revascularized within hours by the process of end-to-end anastomoses of graft and host blood vessels (Abbott, 1942; Abbott, 1947; Deleu, 1965). Regardless of the method of revascularization, the amount of bone regeneration during the first phase depends on the amount of transplanted bone cells that survive the grafting procedure (Ham, 1952; Ray, 1963; Basset, 1972).

Gray (1982) and coworkers focused on the relative osteogenetic contributions of the different cells within cancellous bone grafts during the first 16 days following subcutaneous transplantation in rats. The author found that the endosteum was the most osteogenic element, followed by the periosteum.

The graft bed undergoes changes which lead to a second phase of bone regeneration that begins in the second week and becomes crucial four or five weeks following transplantation (Deleu, 1965). Intense angiogenesis and fibroblastic proliferation from the graft bed begins at the time of grafting and osteogenesis from host connective tissues soon

follows (Enneking, 1975; Chambers, 1980). The importance of the vascular ingrowth is stressed by the recent work showing osteoclasts to be fused with circulating monocytes (Marx, 1946; Chambers, 1980).

The very stimulus to angiogenesis arises from macrophages, platelets and tissue hypoxia (Branemark, 1965; Knighton, 1981). Complete revascularization is also dependent on the graft thickness and the vascularity of the adjacent tissue. Once revascularization is complete, fibroblasts and other mesenchymal cells differentiate into osteoblasts and begin to lay down new bone. Collagen systhesis is stimulated by a slightly acidic environment and may be initiated in hypoxic tissue with tensions as low as 20 torr (Remensynder, 1968; Hunt, 1972). There is evidence that a protein or proteins found within the bone (BMP) may induce these reactions within the surrounding soft tissues of the graft bed (osteoinduction) (Urist, 1970; Urist, 1972) as discussed earlier.

This second phase is also responsible for the orderly incorporation of the graft into the host bed, with continued resorption, remodeling, and replacement. The process of new tissue invading along channels made by invasive blood vessels or along pre-existing channels in the transplanted bone was called "Schleichender ersatz" by Axhausen in describing the bone graft experiments of Barth as cited by Ray (1972). Later, this German phase was translated literally as "creeping substitution" to describe a dynamic reconstructive and healing process for bone transplantation. In summary, the second phase may be viewed as a bone production, maturation, and remodeling phase.

AUTOGENOUS BONE GRAFTING

Autogenous bone grafts have been used frequently since the early 1900's (Chase, 1955) and it is estimated that approximately 200,000 bone grafts are performed in the United States each year (Heppenstall, 1980). Fresh autologous bone is usually considered the ideal bone graft material. It is unique in that it is the only type of bone graft to supply living, immuncompatible bone cells essential to the initial phase of osteogenesis.

The iliac crest is the most common source for these grafts. Other sources include, parietal bone and rib.

Autogenous bone, in both free or composite grafts, offers the following advantages:

1. It provides osteogenic cells for healing.
2. It does not trigger an immunological response.

The disadvantages are

1. The harvesting is sometimes a more invasive procedure than the primary surgical procedure.
2. Requires an additional surgical site as donor.
3. Prolonged hospital stay (Salama, 1983).
4. Overall major complication rate of 8.6 % (Younger,1989).

Complications encountered in each of these donor sites will not be discussed in this paper. These complications are described in detail in the review on bone grafting by Ellis (1989). The following may be seen as a condensed list of complications associated with harvesting bone from the iliac crest; delayed ambulation, paresthesia, adynamic ileus, incisional hernia, hematoma, infection (Ghent, 1971; Challis, 1975; Laurie, 1984).

ALTERNATIVES TO AUTOGENOUS BONE GRAFT

The search for an ideal grafting material that could replace autologous bone graft has lead to numerous studies on various grafting materials. As we mentionned earlier, three major types of graft are available to the surgeon as substitutes for autologous bone graft. We will first review homologous graft and alloplast material before reviewing in detail the xenograft which is the material investigated in this study.

1. Allograft

The allografts represent an interesting alternative to autologous bone graft, however, there are disadvantages to their use. It has been found that they can cause an immunologic reaction as allograft tissues are derived from another individual even though it may be from the same species.

Today most homogenous bone is treated by deep-freezing or freeze-drying. The technique of freeze-drying bone was described by Hyatt and other's in the early 1950's (Hyatt, 1952; Hyatt, 1954; Hyatt,1959). Theoretically, this method relies on cellular destruction within the graft, accompanied by a biochemical effect altering the antigenicity of the osseous matrix of the graft material (Bullingham, 1952). Chalmers found that freeze-drying appeared to inactivate the histocompatibility of antigens in bone homografts (Chalmers, 1959). Quattlebaum in a clinical study found no anti-HLA antibodies to freeze-dried cortical bone allografts in humans (Quattlebaum, 1988). The unprocessed homograft (allograft), however, evokes a very strong rejection response (Anderson, 1961; Langer, 1975). Another concern with the use of allograft is the risk of transmitting disease to the patient.

Transmission of HIV-1 from bone grafts has now been reported from two donnors (cited by Ellis, 1993). Unfortunately, there is usually a period of several weeks to 6 months between HIV infection and the appearance of HIV antibody. In one of the two cases reported, the graft was procured between the time the donor became infected and the appearance of the HIV antibodies. However, the possibility of transplanting a bone allogaft from a donor infected with human immunodeficiency virus (HIV) is remote, provided there is a combination of rigorous donor selection and exclusion, screening for the HIV antigen and antibody, and histopathologic studies of donor tissues (Buck, 1988). The chance of obtaining a bone allograft from an HIV-infected donor who failed to be excluded by the above techniques is calculated to be one in well over a million (Buck, 1988). On the other hand, if adequate precautions are not taken, the risk might be as high as one in 161 (Buck, 1988). Nevertheless, tissue banking has provided surgeons with a readily available, relatively inexpensive, and reasonablely safe selection of homologous bone for clinical use (Hyatt, 1959).

Today, most surgeons currently use homologous banked bone (femoral heads removed during hip replacement surgery) with good results as an alternative to autologous bone (Milinin, 1981; Friedlaender, 1982; Savini, 1986). Three major forms of allograft are available to the surgeon; fresh-frozen, freeze-dried bone, and freeze-dried demineralized bone matrix (FDDB).

Banked bone apparently does not heal as predictably as an autogenous bone graft (Heiple, 1963). In 1968, Urist stated "Neither undecalcified nor decalcified allogeneic bone grafts are rated equal to fresh, warm, autologous bone for repair of large bone defects in adult individuals" (Urist, 1968). That statement has been supported by two animals studies (Burchardt, 1977; Burchardt, 1978).

It is important to understand that any of the treatments required to eliminate the immunologic response will destroy any remaining cell within the graft and, therefore, homologous bone grafts cannot participate in the first phase of osteogenesis. Thus, the host must produce all of the essential elements in the graft bed for a homologous bone graft to become incorporated.

Initially, homologous bone grafts were thought to heal only by osteoconduction. Later, the discovery of bone induction brought the realization that bone is one of the few tissues in the human body that retains the primordial capacity to induce regeneration of lost parts. Today, it is recognized that freeze-dried homologous bone, whether undecalcified or decalcified, has BMP activity (Reddi, 1972). However in undecalcified bone, the protein's action (BMP) is somewhat hindered by bone mineral and decalcification might be necessary to unleash the BMP (Urist, 1970).

Morphometric studies show that demineralized implants do not undergo resorption during bone induction, in contrast to mineral containing powders which are resorbed without bone production (Glowacki, 1981). Thus, healing by induced osteogenesis may bypass the resorption seen with healing of mineral containing grafts (Mulliken, 1981).

Boyne (1973) compared different allograft matrices in combination with autogeneic haemopoietic marrow. The author found that the most effective allograft matrix was the surface decalcified allogeneic cancellous bone. He concluded that certain calcified materials tend to enhance the osteogenic potential of the marrow substrate by providing surface nidus for deposition of new bone and by further apparently inducing the marrow autograft to form new osseous tissue independent of the allograft subtance within the test system (Boyne, 1973).

Finally, Ellis used homologous bone grafts in 135 maxillofacial surgical procedures with satisfactory results (Ellis, 1993).

2. Alloplast

Many different synthetic substances have been used as a substitute to autologous or homologous bone graft including metals, ceramics, carbons, HTR, and calcium phosphates. The alloplastic materials are bioinert and some claim to be bioactive (Osborne, 1980). Bioactive ceramics include hydroxyapatite, fluoroapatites, bioglass and tricalcium phosphates. These ceramic materials themselves are not considered osteogenic (Nade, 1983; McDavid, 1979), but are thought to be osteoconductive once the bone forming process has been initiated (Nade, 1979). This was confirmed by McDavid (1979) who found that with subcutaneous (heterotopic) implants bone did not form about tricalcium phosphate ceramic in the absence of a coating of bone marrow.

Other animal experiments and clinical studies have also shown hydroxyapatite to be biocompatible and osteoconductive (Klinge, 1992; Fukata, 1992). However, this concept is still controversial, Pinholt (1992) tested four commercial apatites used for subperiosteal alveolar ridge augmentation in the maxilla of rats. He found that not one of them had caused osteoinduction or osteoconduction, but had resulted in encapsulation with associated multinuclear giant cells.

The ideal substitute for a bone graft, from a biological and biochemical perspective, is one that is completely resorbed and replaced by new host bone. Previous studies have demonstrated the biocompatibility and biodegradability of three different porous ceramics: calcium aluminate, calcium hydroxyapatite and tricalcium phosphate (Nade, 1983; Uchida, 1984). With regard to biodegradability of ceramics, Bhaskar

(1971) showed that ceramic particules are phagocytized by macrophages and giant cells, and he suggested a cellular mechanism of degradability of tricalcium phosphate other than passive dissolution. However, Uchida (1985) found that the rate of resorption is very slow, so that most of the ceramic was still present six months after implantation. According to the author complete degradation of implanted ceramics may need several years, although he suggested that this may depend on the size of the ceramic implant.

With respect to bone formation, Uchida (1984) found that when ceramics were implanted onto the parietal bone of rat and rabbit skulls, sites primarely devoid of bone marrow, bone formation in and around the ceramics was very limited. When the same porous ceramics were implanted into tibiae of rats and rabbits, significant bone growth was found into the pores of the graft. This was explained by the abundant bone marrow found in the tibia as compared to the skull.

Bone ingrowth into pores of calcium phosphate ceramics has now been observed by numerous investigators using a variety of materials and animal models (Bhaskar, 1971; Nelson, 1977; Winter, 1981). Animal studies have demonstrated that porous hydroxyapatite induced more bony ingrowth than its dense nonporous form (Boyne, 1987). The larger the pores, the higher the rate of bone formation within the pores of the graft (Uchida, 1985).

Because the reliability of bone formation after implantation of hydroxyapatite has not been fully accepted, some investigators have combined autologous bone (Tidwell, 1992; May, 1993) or BMP (Ono, 1992) with hydroxyapatite in order to improve bone formation. Ono (1992) combined hydroxyapatite with BMP for implantation under the periosteum of the rabbit skull. The results of the histopathologic examinations revealed that marginal bone formation was found

in the pores on the surface between the pellets and the skull in the control group. In the bone morphogenetic protein group, on the other hand, very active bone formation was found not only at the interface in contact with the skull but also surrounding the whole pellet.

3. Xenograft (Bovine bone)

Numerous xenograft materials have been used clinically as substitutes for autogenous bone grafts in the past. The first recorded instance of the use of animal bone grafts in humans was reported as early as 1668 (Meekeren, 1954). More than a century ago, Senn (1889) filled post-infectious cavities with chips of decalcified bovine cortical bone. In 1895, Landerer succeeded in replacing the diaphysis of a finger with macerated bone from a dog (cited by Maatz, 1957). Lexer (1924) had reported the use of macerated grafts to fill defects in bone. The terms 'maceration' indicates that the bone was freed of all soft tissue (Lexer, 1924).

In 1935, Orell published a comprehensive study addressing the issue of the use of necrotic bone substances obtained from different animals and their use as a graft material for humans. During the next five years Orell's work was followed by a series of articles (Parisel, 1938; Risinger, 1938; Stark, 1940) and his method was recognized by many surgeons in Central Europe as an alternative to autologous transplantation.

Next bovine bone became a relatively popular graft material during the 1950's and early 1960's in Europe under the name of Kiel Bone. Later, in North America, it was called Boplant. Since these two commercial products had different processing techniques, we decided to review them separately. This review also included two new products, Bio-oss and T-650 Laddec, recently introduced in the market.

In 1957, Maatz and Bauermeister from Kiel, Germany described a method of bone maceration which was used to prepare the bovine bone (Kiel Bone) for different grafting procedures in humans (Maatz, 1957). The authors recommended that the method of maceration must meet four criteria to be considered clinically acceptable.

1. "The deproteinization must be as complete as possible in order to reduce the protein bound incompatibility between host and graft, without however, reducing the biological value of the graft which may be due to the presence of a biocatalyst".

2. "Biological values must not be diminished by the macerating agents".

3. "The mechanical qualities of the graft must not be affected by maceration".

4. "The macerated graft must be sterile".

The first concept described by Maatz and Bauermeister was reinforced by Fushs (1966). He recognized that this protein residue is of great importance since new bone formation decreases as the protein content of the graft decreases (Fushs, 1966).

Investigations on animal models showed that this graft material, when implanted in the muscle of dog, produced callus formation after 15 days (Maatz, 1957). The author cited that "the macerated bone induced metaplasia in the proliferating host tissue and bone was formed". In fact, they had described the osteoinductive properties of the heterograft (Kiel Bone). When the processed bovine bone was implanted in the spongiosa of dog's femur, marked new bone formation had taken place

thoughout the entire graft, in the center as well as at the periphery. Finally they concluded that for the most part, the graft had been replaced by bone tissue from the host. This observation was a description of osteoconductivity and "creeping substitution" into the grafted bovine bone, even if the concept was not clearly defined at that time. In 1961, Maatz reported the use of this macerated bovine bone in more than 200 patients with good results based on a clinical evaluation.

From 1965 to 1966, Churchil-Davidson reported the use of Kiel Bone on 82 occasions in 81 patients for orthopedic operations with a clinical success rate of 80% (Churchil-Davidson, 1967). The Kiel Bone was found to be a highly successful material especially for filling cavities. His results indicated that inlay grafting had been more successful than onlay grafting (Churchill-Davidson, 1967).

In 1972, Taheri and Gueramy prepared a series of 200 cases of anterior cervical spine fusions in which dowels of Kiel Bone were used with good result (Taheri and Gueramy, 1972). Their experience showed that the results with the use of Kiel Bone were equal to those obtained with autogenous bone in a previous study, but without the added morbidity and complications of the donor site operation. Their results were based on radiographic grading and clinical evaluation. As cited by the authors, "It has been found that a heterograft, such as Kiel Bone, which has no osteogenic inductive capacity or osteogenic potency, must be placed in a vascular field and be well immobilized against a cancellous bone bed". Following this publication, two additional reports have been published with respect to the use of Kiel Bone for anterior cervical fusion, again with favourable results (Goran, 1978; Ramam, 1975). Furthermore, in 1982, Siqueria presented a series of 221 patients who underwent anterior cervical interbody fusion using Kiel Bone dowels inserted in the intervertebral space.

On the basis of radiologic and clinical examination, their experience showed that this type of graft was equal to autologous bone graft in terms of fusion and no case of rejection, allergic reaction or non fusion, was observed.

More recently, Binderman (1987) compared the healing achieved in large gaps in the long bone of rats both untreated (controls) and when packed with heterograft (Kiel Bone) or synthetic graft (HTR polymer) materials, with and without added bone marrow or calcium hydroxide (Ca (OH)2). The animals were sacrificed at 36 days. When Kiel Bone was used alone to filled the gap, a more dense fibrous tissue surrounded the Kiel Bone particules. Many macrophages and fewer multinucleated cells (osteoclast-like) were seen in the near vicinity of the Kiel Bone. In the early stage of bone healing (36 days) the results with Kiel Bone were poor, but this situation may have improved at a later time. However, when Kiel Bone was mixed with autologous bone marrow, this combination induced bone formation in the gap, and a solid bridge was obtained at 36 days. The result of this study demonstrated that bone marrow seems to promote ostegenicity when combined with Kiel Bone.

Combining xenograft and fresh autologous bone marrow is not a new method and has been reported in the literature (Salama, 1978; Plenk, 1972; Salama, 1973). It has been repeatedly stated that autologous red bone marrow can stimulate the formation of bone (Burwell,1964; Richter, 1968; Boyne, 1969). Moreover, this osteogenic capacity of fresh autologous marrow can be transferred not only to marrow free autologous and homologous bone graft (Burwell,1964), but also to various types of stored bone (Burwell,1966a). A composite of graft bone and marrow seems to form considerably more new bone than do either of the components of the graft transplanted separately (Burwell 1964).

Even before the work of Binderman (1987), Plenk (1972) considered that Kiel Bone had lost its osteogenic potential because of preserving and denaturing procedures, so that bone formation could not be induced. This hypothesis was supported by the fact that implantation of processed heterograft (Kiel Bone) into a bed of connective or muscular tissue, had not been successful in inducing bone formation (Heiple, 1963). This demonstrated results contradicting those of Maatz (1957). Plenk (1972), investigated whether osteogenic capacity could be imparted to Kiel Bone by supplementation with fresh autologous marrow. Again when Kiel Bone alone was placed in a 5mm osseous defect in the ilium of rats, no bone formation occured, and the graft was encapsulated by connective tissue. Whereas, when it was impregnated with autologous marrow, new bone formation was induced.

As pointed out by Burwell (1966), the lack of osteogenic potency in preserved bone may be corrected either "by natural invasion of the implant by bone forming cells at the site of implantation" or "by artificial impregnation of the implant with bone forming cells before implantation" (Burwell, 1966). However, the first of these possibilities may not be sufficient to bridge a large osseous defect since Kiel Bone grafts alone have not succeeded in bridging osseous defects in the human jaw (Hollman,1970).

In 1960's, Unilab* acquired the original protocol for the processing of calf bone (Kiel Bone). Since then, the manufacturer (Unilab) is now using mature bovine bone instead of calf bone. The manufacturer has also changed part of the chemical processing and now provides a more sophisticated pyrogen testing protocol as well as an improved sterilization technique utilizing ethylene dioxide (Salama, 1983). The manufacturer has not, however, tested its product with the same diligence as performed previously.

* **UNILAB SURGIBONE** 764 RAMSEY, HILLSIDE, N.J. USA 07205

Since 1975, the Canadian Government gave marketing approval for Unilab Surgibone. The commercial product available today consists of partly deproteinized bone prepared from freshly killed mature cows. According to the manufacturer, the graft contains a protein content of approximately 20% to 29% and the chemical composition is that of an hydroxyapatite, approximated by the formula Ca3(Po4)2 Ca(OH)2. The calcified, rehydrated Unilab Surgibone reveals a well preserved structure composed mainly of collagen fibers with a measured periodicity of between 600 and 700 A.

While Kiel Bone was being developed in Europe, Anderson (1959) in North America was also interested in heterogenous bone grafts. This author has shown that small fragments of heterogenous bone grafts, in the form of a paste, were penetrated by blood vessels as rapidly as were the large fragments of autogenous cancellous grafts when grafted into the anterior chamber of the rat's eye.

In 1961, encouraged by the initial results, Anderson (1961) transplanted small fragments of cancellous calf bone, preserved in various ways, into the anterior chamber of the rat's eye. These xenografts were neither deproteinized or processed before implantation. The results were discouraging; no evidence of new bone or any semblance of osteogenesis was noted throughout the study, instead they did produce a host inflammatory, foreign body and fibroblastic reaction. The author attributed the poor result to the non processed form of bone, or more specifically, it's antigenicity as related to the presence of protein.

In 1964, a new type of processed heterogenous bovine bone (Boplant) for grafting procedures became commercially available following animal studies and clinical trials (Harmon, 1964; Karges, 1963). Processed heterogenous bone (Boplant) was furnished by Ralph Meiser of the Squibb

Institute for Medical Reseach, New Brunswick, NJ. The method of preparation of Boplant was different from that previously described for processing Kiel Bone (Karges, 1963).

Karges (1963) investigated the response of dogs to implants of the same type of processed calf bone. Both cancellous and cortical implants were used, and the duration of the experiment varied from two weeks to six months. Fresh autografts, in the same animals, provided a basis for comparison. With both compact and cancellous heterogenous implants, no inflammatory reactions were noted. In the cancellous implants, new bone surrounded the trabecular bone by one month, and at the end of the three months only ten to twenty percent of the implant remained. These authors considered that "at one month and thereafter, new bone formation in the heterograft was certainly equal to that of the autograft, and in no instance was there evidence of rejection" (Karges, 1963).

In 1964, Anderson published a series of clinical trials using Boplant in various orthopedic procedures (Anderson,1964). Thirty-six patients were grafted with boplant and biopsies were taken at various times, ranging from two weeks to nineteen months. The heterogenous bone transplants showed no histologic evidence of an immune reaction but readily evoked a human host osteotogenic response. Revascularization occured by two weeks in the cancellous heterograft. Progressive replacement and incomplete remodeling occurred in the cancellous heterograft by six months.

Although some reports confirmed the successful clinical use of Boplant in orthopedic, rhinological, plastic and reconstructives procedures (Anderson,1965; Ellis, 1965), other investigations (Kramer, 1968) and clinical trials have shown poor results with the use of Boplant (Pieron, 1968; Hughes,

1968; Bell, 1968).

Kramer in 1968 tested Boplant for use as onlay and inlay grafts implanted into calvarial defect (8mm) of rabbits. The results with the Boplant onlays were similar to those with the inlays. There was an early massive infiltration with polymorphonuclear leucocytes, followed by continuing chronic inflammation, absorption of the implant and little or no new bone formation. In the onlay experiment the Boplant was replaced by a small fibrous nodule.

A year before, Heiple (1967) created standard segmental defects in the ulnar bones of dogs. In each animal, the defect on one side received an autograft and the defect on the other side received augmentation with Boplant. Control animals had the same defect but no graft was inserted. Heiple and his colleagues commented that the stricking feature of the early phases of healing, following the introduction of Boplant, was marked by an inflammatory response. As the inflammation subsided two weeks later, the spaces within the implant became filled with dense fibrous tissue. During the first few weeks there was virtually no apposition of new bone within the implant. Absorption of the Boplant was evident by the second week and, after three months, only small fragments remained.

Boyne and Luke (1967) examined the response to "second generation" of Boplant implanted in dogs. The "second set" heterografts were rejected by the fourth month in fifteen animals, whereas all the control autografts were retained in the contralateral sides of the same animals. A chronic inflammatory cell infiltration persisted around residual fragments of the Boplant.

In 1968, Pieron reported a series of 33 patients grafted with Boplant for various orthopedic procedures, mainly lumbar

spine fusion (Pieron, 1968). Their findings at surgical re-exploration were disappointing. Re-exploration was done in nine of the twelve patients in which the primary operation had failed to achieve fusion. In nearly all cases the pieces of Boplant were found lying free, without any bony union. An overall performance that forced the surgeons to abandonned the Boplant.

Periodontists became interested in Boplant for its ability to repair bone defects occuring in the human oral cavity as a result of chronic inflammation around the teeth (periodontitis). Scopp (1966) presented a clinical trial in which 56 patients were grafted with Boplant to correct 77 periodontal oral defects. He hypothetized that the prime source of osteoblasts for the graft regeneration was from the surrounding bone. Accordingly, a successful procedure, he felt, was related to the bony surface area. The more existing bony walls, the greater the potential for bone filling the defect. The author also emphasized the fact that a good soft tissue surrounding the graft would contribute to the blood supply and would provide extra-cellular fluids for nutrition of the migrating osteoblasts. The results obtained with Boplant compare favorably with those they have obtained with autogenous bone grafts.

More recently Pinholt (1991) tested an other commercial form of xenogenic bone (Bio-oss) in rats. Since Bio-oss was reported to initiate new bone formation, Pinholt tested Bio-oss for alveolar ridge augmentation by osteoinduction (Boyne, 1987). To confirm its possible osteoinductive properties the material was also implanted heteropically in the abdominal muscles of rats. Light microscopic examination revealed no osteoinduction or osteoconduction in connection with sintered or unsintered Bio-oss. Foreign body reaction was observed around both forms at four weeks. The authors commented that an increased observation time might have revealed the

substitution of the implanted Bio-oss by osteoconduction.

Fukata (1992) created full thickness skull defects in rabbits and found that Bio-oss alone did not achieve full bone formation in the entire area of the skull defect. However, he claimed that Bio-oss had good osteoconductive properties.

In summary, the previously descibed products (Kiel Bone, Boplant, Bio-oss) are considered by the majority of investigators cited in this review as being an osteoconductive material. However, the T-650, Laddec is the newest commercially available xenograft and is claimed to be more osteogenic when compared to Unilab Surgibone (Chappard, 1991).

Chappard (1991) in his preliminary study investigated the biocompatibility of this new highly purified xenograft in the rabbit model by histological methods. The T-650 was found to drastically induce osteogenesis according to the author. The rabbit's condyle implanted with Unilab Surgibone showed a fibrous stroma surrounding the implant fragments with the presence of osteoclasts and resorption but with little bony formation. However, no foreign body reaction was observed.

The Unilab Surgibone was found to be less osteogenic in this experiment when compared to T-650. Although the composition of the Unilab Surgibone is somewhat similar to the T-650 (29 % collagen protein versus 27 % for the T-650), the author attributed these results to the purification technique of the T-650 which prevents denaturation of the "mineralo-collagenic matrix". The Unilab Surgibone is thus considered by the author as well purified (because no immune reactions occured) but less osteogenic because of the denaturation of the protein probably occuring during the processing technique.

The T-650 has been used in one clinical study (Missika, 1993). Following the extraction of a tooth, an immediate

endosseous implant was placed in the extraction socket. A space between the implant and the alveolar bone or a buccal dehiscence was present. The T-650 was packed into the defect and the mucoperiosteal flap was closed in the usual manner. The results were based on clinical impression at the second surgical phase. For nine out of eleven patients, the osseointegration was found to be adequate clinically.

The mechanical properties of this new product were tested in another investigation (Poumarat, 1993) and were found to be comparable to those of human bone.

a. Antigenicity

One of the primary questions arising with the use of heterogenous bone is the immunological response. It is believed that T-Cell responses are the most important factors in xenogenic bone transplantation (Burwell, 1964; Burwell, 1985).

The antigenic potential of xenografts is greater than of homologous bone (Delustro, 1990). Because the organic matrix of xenogeneic bone is antigenically dissimilar to that of human bone, heterogenous grafts must be scrupulously treated to prevent graft rejection. In fact, the unprocessed heterograft will evoke a very strong rejection response (Anderson, 1961; Enneking, 1972). However, treatments to reduce immunogenicity of the graft may destroy properties deemed desirable in the graft (Maatz, 1957; Tuli, 1979). The chemically treated graft will reduce the antigenicity, but these procedures may alter any osseoinductive effect, perhaps by denaturing bone morphogenic proteins within the bone.

In 1974, Elves studied a different form of deproteinized bovine bone and found that the lowest incidence of antibody

formation was found in rats receiving grafts containing fully deproteinized bone (Elves, 1974). The partially deproteinized "Kiel Bone" gave rise to more immunological reactions when compared to the fully deproteinized "Oswestry bone". The authors also established a correlation between the immunological response and new bone formation. The highest levels of new bone formation were found in the "Oswestry bone" which was the least antigenic at two weeks after implantation in the rat.

In 1978, Salama examined the immune response to combined xenografts of Kiel Bone and autologous marrow by serological methods in 28 patients and the leucocytic reaction in the peripheral blood in 15 patients. The results showed that Kiel Bone was very weakly antigenic in the human host (Salama, 1978b).

More recently new methods of processing bovine bone have been tested in rabbits and were found to promote significant bone formation without any immunogenic reaction (Ma, 1989; Chappard, 1991).

MATERIALS & METHODS

EXPERIMENT I : Unilab Surgibone inlay graft

Thirty male Sprague-Dawley rats weighing 310-340 grams were anesthetized with intraperitoneal injection of 0,15 ml per 100 grams body weight of nembutal (50 mg per ml), diazepam (5mg per ml) and saline (1:2:1 by volume). The anesthetized rats were placed in a supine position and secured on an animal mount. The field was kept as sterile as possible and all surgical techniques were designed to replicate those of the clinical setting.

The right hind limb was immobilized, cleaned with proviodine, shaved and drapped with sterilized surgical paper to isolate the hind limb from the body. A 15 mm incision was made over the prominence of the tibia from a point just inferior to the knee joint. Using blunt dissection care was taken to displace the muscles from the anterior tibia to expose the underlying periosteum. The periosteum was incised and reflected to expose the anterior surface of the tibia. The tibial tubercule was identified and served as a reference point for graft placement.

Using an electric hand piece with a 703 fissure bur under copious saline irrigation, a groove measuring 2 mm wide, 3 mm deep and 5 mm long was made in the rat tibia starting at the tubercule continuing proximally. The resultant defect had three intact bony walls, which did not jeopardize the strength of the tibia.

Sterile bovine bone granules were packed into the defect (approximately 0,012 to 0,015 gram of bone was used for each defect). Closure of the wound was achieved in layered fashion using 4-0 dexon for the muscle and skin.

On the same rat a control was established using the contralateral leg. A similar procedure was performed on the left tibia. A groove of identical measurements and location was made in the tibia; however, the defect was not packed with bone. Closure of the wound was achieved in the same manner as the contralateral side. Animals were evaluated for periods up to 32 weeks, during which time they were monitored daily by visual inspection for infection, wound dehiscence and gait. A time schedule was established and animals were sacrificed by intracardiac perfusion with 5 % gluteraldehyde in cacodylate buffer solution (appendix 1) weekly for eight weeks and then at 16 and 32 weeks. A total of ten groups (three rats per group) were evaluated using this method.

Following sacrifice, soft tissues were dissected off of the bone, the tibia was disarticulated at the knee joint and the distal acpect of the leg was cut off above the ankle with bone forceps. Some soft tissue was left over the area of the graft so that it could be examined. The specimen was then immerse fixed for three hours in 5 % gluteraldehyde, washed in sodium cacodylate buffer, and decalcified in 4,13 % disodium EDTA for eight weeks (Warshawsky and Moore, 1967; appendix 1).

The decalcified specimens were thoroughly rinsed in cacodylate buffer and the grafted areas \ control defects were sectioned into three pieces using a sharp thin blade. Each piece was post fixed in 1 % osmium tetroxide for two hours, dehydrated through graded concentrations of acetone, and embedded in Epon 812 (appendix 1). The embedded blocks were trimmed using a dental handpiece and a separating disk to identified the edge of the specimen. Thick sections (1,0 micron) were cut with a diamond knife, stained with toluidine blue and visualized by light microscopy.

Preliminary morphometric analysis using the "hit method" was imployed to measure the relative size of the grafted

Unilab Surgibone granules at two different time intervals (week 1 and 32).

This was performed by counting the numbers of intersection points visualized from an ocular grid that crossed over the granules at one hundred magnification, by means of light microscopy.

EXPERIMENT II : Unilab Surgibone onlay graft

Thirty male Sprague-Dawley rats weighing 320-360 grams were used. The anesthesia, prepping and surgical techniques to expose the tibia were performed in the same manner as for experiment I. Once the tibial tubercule was identified, three holes (1,0mm diameter) at 0,5 - 1,0 mm intervals were made linearly in the bone starting at the trabecule and moving proximally. The surgical bur mounted on the dental hand piece perforated the cortical bony surface until bleeding had occurred. Drilling was performed under copious irrigation consisting of normal saline.

A sterile cancellous block of Unilab Surgibone previously trimmed and measuring 2.5 mm. wide by 2.5 mm. deep by 5 mm. long was then placed on top of the three perforations and secured by means of a circum-tibial 4-0 dexon suture. This technique allowed the grafted cancellous block to be exposed directly to the underlying bleeding marrow via the three holes.

Once the block had turned red, indicating good bleeding from the cortical perforations, closure was achieved in layers as described for experiment I. The animals were sacrificed and processed for histological evaluation in the same manner as in experiment I. The "hit method" was also employed to measure resorption of the onlay graft at different time intervals (weeks 1 and 32).

RESULTS

EXPERIMENT I : Unilab Surgibone Inlay graft

At one week post-surgery, the grafted granules were surrounded by a significant amount of woven bone (Figure 1a). Osteoblasts were seen forming new bone next to the implant surface and a cementing line was present at the interface implant - woven bone (Figure 1b). Marrow elements and vascular channels were also seen close to the implant. There was no sign of a foreign body reaction. The old haversian canals of the bovine bone implant were already invaded with vascular channels and osteoblasts forming new bone into the canal. However, the lacunae of the implant remained empty (Figure 1c). The control leg demonstrated a remarkable repair process characterized by woven bone formation filling the entire defect (Figure 1d).

At the two weeks post-surgery, osteoblasts still appeared active and new bone was seen bridging implants together in some areas (Figure 2a). The marrow elements were reforming in between the new bone and the implants (Figure 2b). The control leg demonstrated new trabeculated bone and the spaces were filled with numerous marrow cells (Figure 2c).

At three weeks post-surgery, the histological features were essentially the same as at two weeks, showing new bone with numerous osteocytes next to the surface of the bovine bone implant (Figure 2d). The granules located at the superior aspect of the defect were exposed to fibrous tissue, in which an occasional osteoclast was identified resorbing the implanted granules (Figure 3a). The control leg demonstrated a thin layer of new bone bridging the superior aspect of the defect. Deeper into the defect, the marrrow elements were evident, replacing the new bone (Figure 3b).

Between *four and seven weeks* post-surgery, the tissue response demonstrated a relative maturation of the findings previously presented. There were considerable amounts of new bone found bridging the implanted granules. This bridging phenomenon appeared to be seen most frequently during this time period (Figure 3c & 3d). The overall picture showed an increase thickness of the superior aspect of the defect (Figure 4a & 4b). However, the thickness was not regular along the defect as many areas were invaded by fibrous tissue rather then new bone (Figure 4c). The increased volume of bone was composed of a mixture of woven bone, implant granules and small medullary cavities containing numerous vascular channels. During this time period a remodeling process of the implant was also noticed. Resorption of the implant and replacement with new bone was observed (Figure 4d).

During that same time period (four to seven weeks), the control leg demonstrated a complete repair of the superior cortical defect by new bone which returned to its original thickness. The medullary cavity was filled entirely with marrow and fat cells (Figure 5a). The trabeculated bone was completely resorbed inside the medullary cavity, so that the thickness of bone was much smaller when compared to the grafted leg of the same time interval (Figure 5a & 5b).

AT eight weeks post-surgery, the osteoblastic activity appeared to have slowed down. The granules were surrounded by lamellar bone. Osteoblasts appeared elongated and thinned out suggesting decreased activity (Figure 5c).

At sixteen weeks post-surgery, the thickness of bone appeared to have decreased. The marrow elements seems to have migrated and replaced new bone thus decreasing the thickness of the bone bridging granules (Figure 5d). The remodelling process observed at 16 weeks was even more evident at 32 weeks. At that time interval, the thickness of the cortical

bone had decreased significantly. When comparing the control to the grafted leg at the same time interval (32 weeks), the thickness of bone at the superior aspect was approximately the same (Figure 6a & 6b). Some of the granules of bovine bone were still engulfed in the superior aspect of the grafted leg (Figure 6c). The medullary cavity demonstrated a more or less normal appearance. Remaining isolated granules which were identified in the medullary cavity were surrounded by a thin residual layer of bone (Figure 6d).

The results of the morphometric analysis are presented in Table 1.

Because the numbers of granules implanted into the tibia varied significantly from one specimen to another, an average number was established per specimen; the total number of "hit points" counted was divided by the number of granules observed within a section. An estimate of the relative resorption was calculated to be 47,2 % at 32 weeks, when compared to the size of the granules at one week.

Due to the small number of specimens that were measured, the data was not analyzed statistically. Thus, the values must be viewed as approximates.

Table 1: Inlay graft

Average size of the granules at one and 32 weeks.

[illegible]	1 WEEK	32 WEEKS
I	12.3	7.6
II	19.9	8.8
III	10.8	6.3
Total	**43.0**	**22.7**

Figure 1:

A - At one week following implantation of the bovine bone, the granules (G) were already surrounded by woven bone (W). Host cortical bone (H).

1.0 μm Epon section. Toluidine blue. x 100.

B - Higher magnification showing a cementing line (arrows) between the granule (G) and the woven bone (W). (one week)

1.0 μm Epon section. Toluidine blue. x 400.

C - Old haversian canal of the bovine bone granule invaded by osteoblasts (arrows), woven bone (W) and vascular channels (v). The granule demonstrated empty lacunae (arrow heads). (one week)

1.0 μm Epon section. Toluidine blue. x 300.

D - Control leg at one week showing fibrous tissue (F) at the superior aspect of the defect through the host cortical bone (H). Woven bone (W) has formed into the defect above the marrow (M).

1.0 μm Epon section. Toluidine blue. x 40.

A

B

C

D

Figure 2:

A - At two weeks, the granules (G) were bridged by new bone (N).

1.0 μm Epon section. Toluidine blue. x 100.

B - Marrow elements reformed between granules (G) at two weeks.

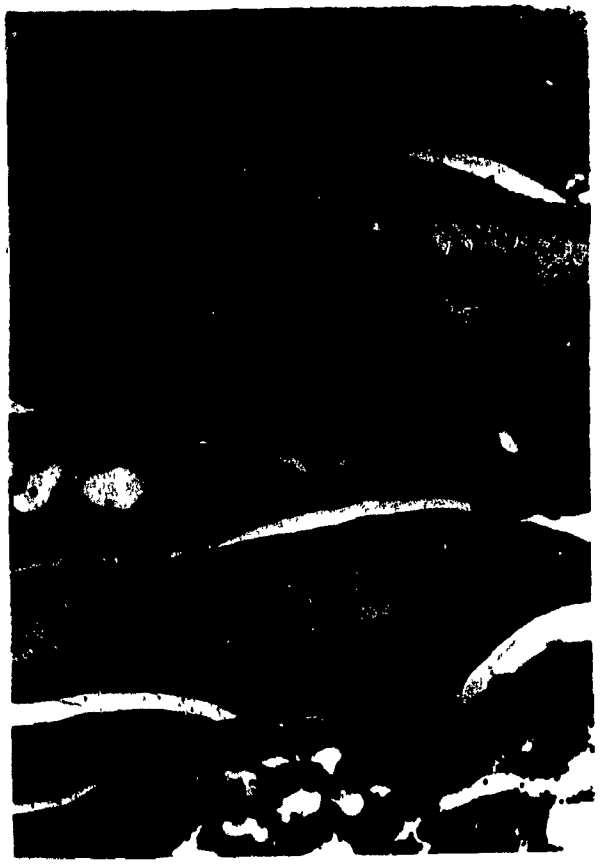
1.0 μm Epon section. Toluidine blue. x 100.

C - Control leg at two weeks showing fibrous tissue (F) and new bone (N) at the superior aspect of the defect.

1.0 μm Epon section. Toluidine blue. x 100.

D - New bone (N) next to the granule (G) showing numerous osteocytes (arrows) and a cementing line (arrow head).

1.0 μm Epon section. Toluidine blue. x 400.



A

B

C

D

Figure 3:

A - Three weeks following implantation, high power magnification showing an osteoclast next to the bovine bone granule (G) surface.

1.0 µm Epon section. Toluidine blue. x 1000.

B - Control leg at three weeks showing new bone (N) at the superior aspect of the defect (arrows).

1.0 µm Epon section. Toluidine blue. x 100.

C - Six weeks following implantation, showing good incorporation of the granules (G) by new bone (N).

1.0 µm Epon section. Toluidine blue. x 100.

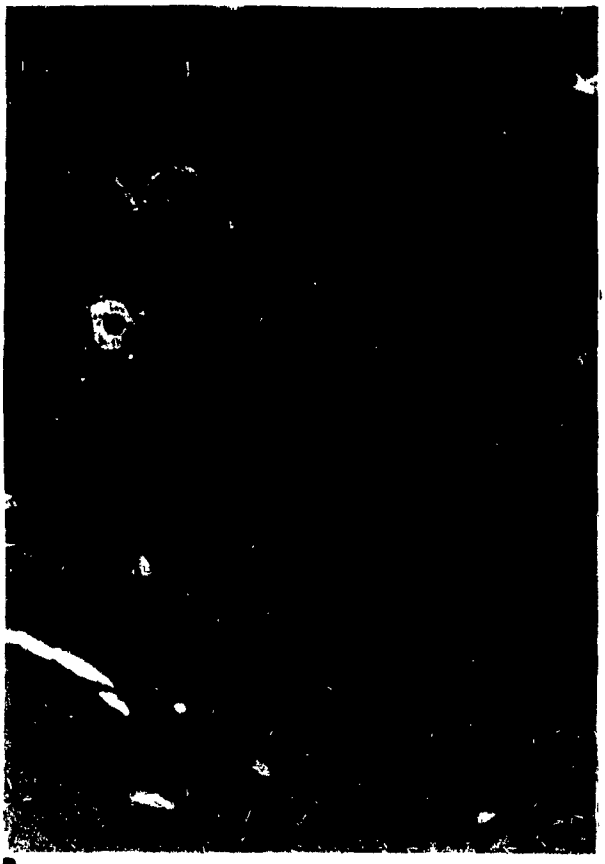
D - Higher magnification of figure 3-C showing new bone (N) bridging two granules (G).

1.0 µm Epon section. Toluidine blue. x 400.

A

B

C



D

Figure 4:

A - B Low power magnification showing the increased thickness of bone at the superior aspect of the defect. (figure A - 5 weeks & figure B - 6 weeks)

1.0 μm Epon section. Toluidine blue. x 40.

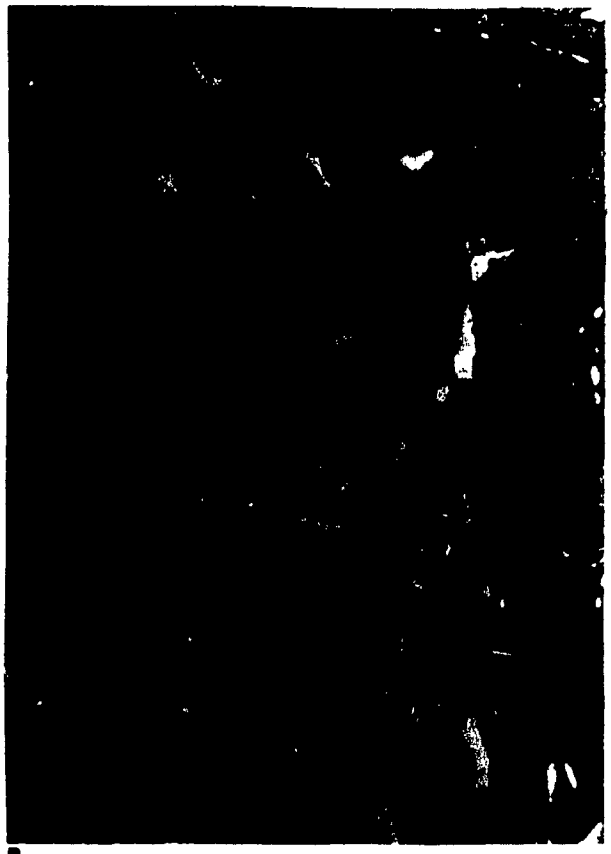
C - Irregularities in the thickness of bone represented by a granule (G) surrounded by fibrous tissue (F). (6 weeks)

1.0 μm Epon section. Toluidine blue. x 40.

D - Five weeks following implantation showing a granule (G) being resorbed by osteoclasts (arrow heads) while osteoblasts (arrows) were forming new bone (N) in the same area.

1.0 μm Epon section. Toluidine blue. x 400.

A



B

C

D

Figure 5:

A - Control leg at seven weeks.

1.0 μm Epon section. Toluidine blue. x 100.

B - Seven weeks following implantation showing increase thikness when compared to figure 5-A.

1.0 μm Epon section. Toluidine blue. x 100.

C - Eight weeks following implantation, showing a granule (G) surrounded by lamellar bone (L) and osteoblasts (arrow).

1.0 μm Epon section. Toluidine blue. x 400.

D - At 16 weeks following implantation of the granules, new bone was replaced by marrow (M) leaving a thin bridge of bone between the granules.

1.0 μm Epon section. Toluidine blue. x 50.

A

B

C

D

Figure 6:

A - B At 32 weeks, the tickness of bone was approximately the same when comparing the grated leg (figure A) to the control (figure B).

1.0 µm Epon section. Toluidine blue. x 100.

C - 32 weeks following implantation showing remodeling of the medullar cavity.

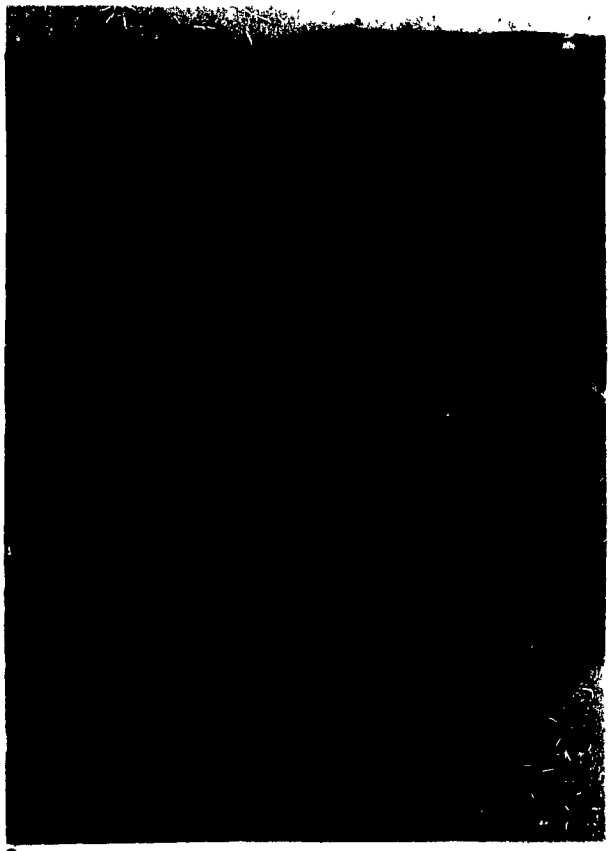
1.0 µm Epon section. Toluidine blue. x 40.

D - Isolated granules (G) in the marrow cavity surrounded by thin layer of bone.

1.0 µm Epon section. Toluidine blue. x 40.

A

B



C

D

EXPERIMENT II : Unilab Surgibone Onlay graft

At one week, the hole created through the cortical bone was clearly identified and filled with woven bone. (Figure 7a). The spaces between the trabeculae of the graft were invaded by loose fibrous tissue (Figure 7b). The lateral aspect of the graft also showed islands of cartilage with hypertrophic cells (Figure 7c). The control leg demonstrated diffuse woven bone formation within the marrow cavity (Figure 7d).

At two weeks, the beginnings of endochondral ossification were identified in the islands of cartilage at the lateral aspect of the graft. A thin layer of fibrous tissue separated this activity from the graft (Figures 8a & 8b). The center of the graft demonstrated fibrous tissue with numerous vascular channels (Figure 8c). The control leg showed a partial repair of the superior aspect of the hole with woven bone.

At three weeks, bone formation was proceeding from the lateral aspect of the onlay towards the center of the graft (Figures 8d & 9a). This invasion of new bone through the graft was seen only at the inferior aspect of the bovine bone implant. By this stage, the control leg demonstrated complete repair of the holes with woven bone (Figure 9b).

A similar tissue response was seen thereafter, such that *weeks four to eight* demonstrated what can be best described as callus formation on the lateral aspect of the onlay graft. Trabeculated bone formed meshwork of intercommunicating spaces which were filled with bone marrow elements (Figure 9c). At the inferior aspect of the onlay graft some areas exhibited bone formation directly against the graft, whereas other areas, showed a thin layer of fibrous tissue which separated the graft from the new bone (Figure 9d). The overall picture shown was of a graft incorporated by new bone at the inferior

aspect (Figures 10a & 10b), and the remaining portion, which represented approximately one quarter of the graft, remained encapsulated by fibrous tissue (Figure 10c). Remodeling of the graft was evidenced by numerous osteoclasts resorbing the implant while osteoblasts were seen forming bone next to the site of osteoclastic activity (Figure 10d). The control leg demonstrated complete bony repair of the holes.

At sixteen weeks, the thickness of the bone was greater in the grafted leg when compared to the control leg of the same time interval (Figures 11a & 11b). Resorption of the graft was even more obvious since small isolated fragments were found engulfed into new bone at the inferior aspect of the graft (Figure 11c).

At 32 weeks, approximately three quarters of the onlay graft was encapsulated in a fibrous nodule (Figure 11d) in which the remaining graft trabeculae appeared thinned out. Below this nodule, at the inferior aspect of what was the original graft, the implant was partially resorbed and replaced by new bone. This produced a significant increase in the thickness of the resultant bone when compared to the control leg (Figures 12a & 12b) & (Figures 12c & 12d).

An analysis of the residual thickness of the graft was calculated. The results comparing graft thickness at one and thirty-two weeks are presented in Table 2.

Table 2: Onlay graft

Relative size of the implant at one week and 32 weeks.

Specimens	1 week	32 weeks
I	153	65
II	151	17
III	122	39
Total	**426**	**121**

The result indicated that the onlay graft appeared approximately 3,5 times smaller at 32 weeks when compared to the first week (Table 2).

Figure 7:

A - One week following implantation showing part of the bovine bone implant (I) sitting on the host cortical bone (H) and over the defect (D).

1.0 μm Epon section. Toluidine blue. x 48.

B - One week following implantation showing invasion by fibrous tissue (F) between trabeculaes of the implant (I).

1.0 μm Epon section. Toluidine blue. x 52.

C - One week following implantation showing chondrocytes (C) at the lateral aspect of the implant (I) near by the host cortical bone (H).

1.0 μm Epon section. Toluidine blue. x 60.

D - Control leg at one week showing the defect filled with new bone (N).

1.0 μm Epon section. Toluidine blue. x 100.

A

B

C

D

Figure 8:

A - Two weeks following implantation showing a callus (arrow heads) formation at the lateral aspect of the onlay graft (I).

1.0 µm Epon section. Toluidine blue. x 40.

B - Higher magnification of figure 8-A showing the callus formation. (2 weeks)

1.0 µm Epon section. Toluidine blue. x 100.

C - Two weeks following implantation showing invasion of fibrous tissue (F) and vascular channels (V) in the center of the implant (I).

1.0 µm Epon section. Toluidine blue. x 100.

D - Three weeks following implantation of the onlay graft showing invasion of new bone (N) between the trabeculaes of the graft (I).

1.0 µm Epon section. Toluidine blue. x 100.

A

B

C

D

Figure 9:

A - Higher magnification of figure 8-D showing osteoblasts (arrow heads) forming new bone between the trabeculae of the graft (I). (3 weeks)

1.0 μm Epon section. Toluidine blue. x 150.

B - Control leg at three weeks showing complete repair of the defect.

1.0 μm Epon section. Toluidine blue. x 40.

C - Four weeks following implantation of the onlay graft demonstrated formation of new bone (N) and small marrow cavities (M) at the lateral aspect of the graft.

1.0 μm Epon section. Toluidine blue. x 100.

D - A thin layer of fibrous tissue (F) was interposed between the graft (I) and the new bone (N). (4 weeks)

1.0 μm Epon section. Toluidine blue. x 400.

A

B

C

D

Figure 10:

A & B - Seven weeks following implantation of the onlay graft showing incorporation of the inferior aspect of the graft (I) by new bone (N).

1.0 μm Epon section. Toluidine blue. x 100.

C - Encapsulation of the superior aspect of the graft (I) by fibrous tissue (F). (6 weeks)

1.0 μm Epon section. Toluidine blue. x 130.

D - Seven weeks following implantation of the onlay graft showing osteoblasts (arrows) and osteoclasts (arrow heads) next to the implant (I) surface.

1.0 μm Epon section. Toluidine blue. x 375.

A

B

C

D

Figure 11:

A - Increased thickness (arrows) of bone at the inferior aspect of the graft.(16 weeks)

1.0 μm Epon section. Toluidine blue. x 40.

B - Control leg showing the thickness (arrows) of bone in comparison to figure A. (16 weeks)

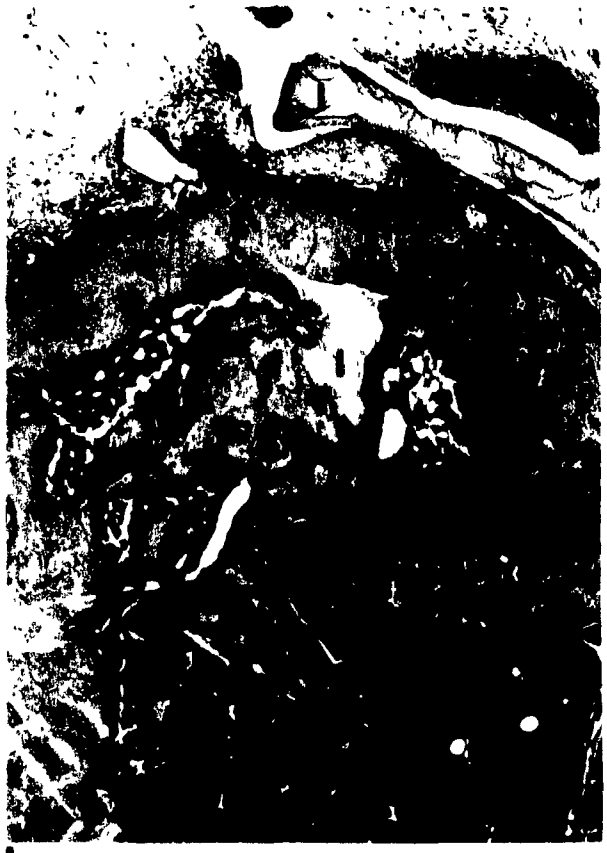
1.0 μm Epon section. Toluidine blue. x 40.

C - Sixteen weeks following implantation of the graft showing an isolated fragment of implant (I) engulfed into new bone (N).

1.0 μm Epon section. Toluidine blue. x 100.

D - Encapsulation by tissue (F) and thinning out of the graft trabeculaes (arrow heads). (32 weeks)

1.0 μm Epon section. Toluidine blue. x 40.



A

B

C

D

Figure 12:

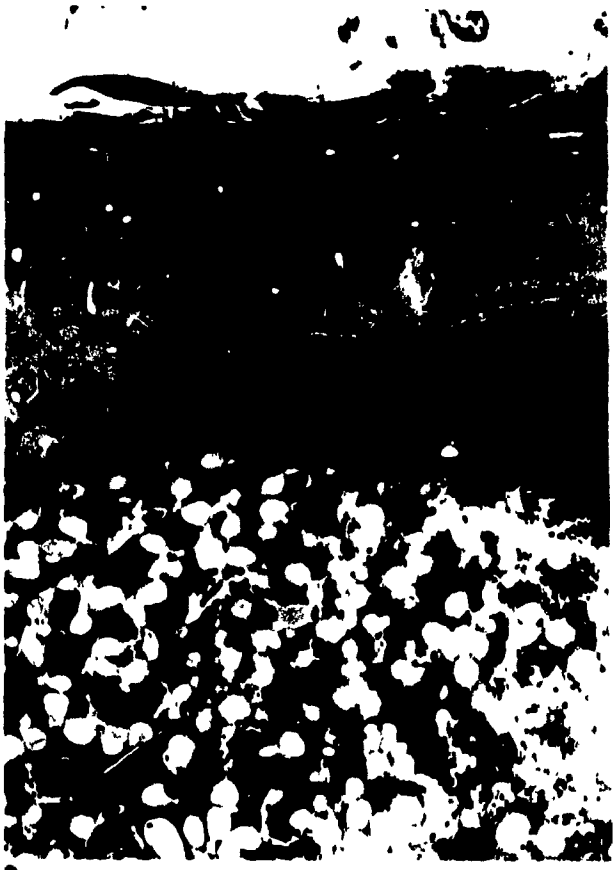
A - B Comparison of the thickness of bone between the grafted leg (figure A) and the control leg (figure B) at 32 weeks.

1.0 µm Epon section. Toluidine blue. x 100.

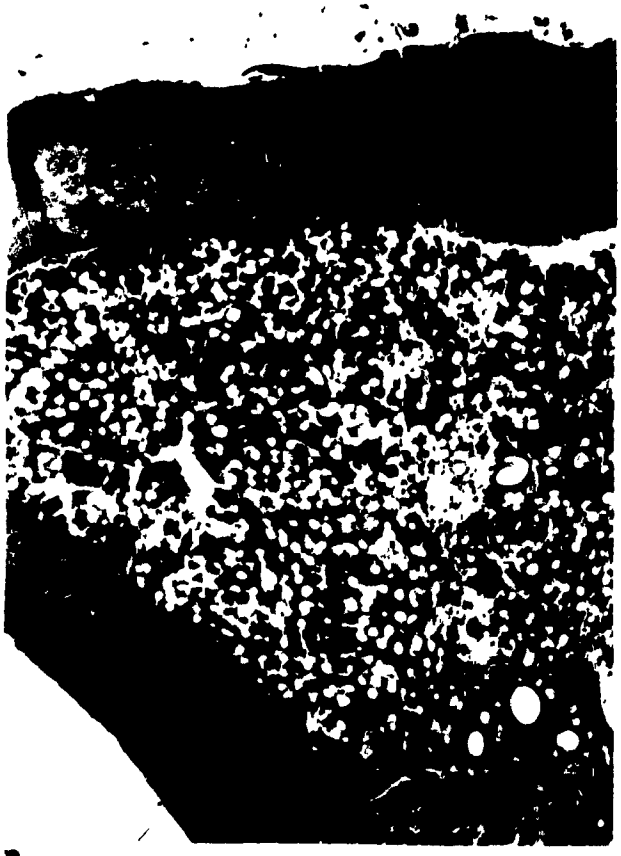
C - Control leg at 32 weeks.

1.0 µm Epon section. Toluidine blue. x 40.

A



B



C

DISCUSSION

The insertion of endosseous dental implant is a relatively simple surgical procedure when the quality and quantity of bone are adequate. The frequent lack of bone in the maxilla and mandible, as previously discussed, justify augmentation procedures prior to placement of titanium implants. Disadvantages associated with the procurement of autogenous bone to augment these deficient areas have been reviewed.

The search for an ideal grafting material that could replace autologous bone has lead to several investigations. The alternatives include a variety of allogenic grafting materials, none of which are comparable to the autologous bone. That is to say, since these materials do not contain cells with osteogenic potential, one cannot assess the first phase (that of osteogenesis) of bone healing about these materials, and for this reason they are inferior to autologous bone as graft materials. With this understood, a bovine bone is a readily available product, it does not transmit disease and demonstrates an osteoconductive potential superior to that of other alloplastic materials (Klinge, 1992). Furthermore, allogenic bone involves complicated bone bank procedures and there is still the potential of transmitting viral disease (Buck, 1988).

The purpose of this investigation was to develop an animal model that will simulate a clinical situation where an onlay and inlay grafting procedure will be used. Bovine bone grafts were then evaluated histologically. The inlay model can be related to a maxillary sinus augmentation procedure, whereas the onlay model can be seen to reproduce the situation at the posterior aspect of the mandible when augmentation is required prior to placement of a dental implant.

The basic histological data obtained will be used for a future experiment which would assess the tissue response to titanium implants placed in the bovine bone graft material. We will discuss the inlay and onlay graft results as separate entities as they demonstrated different behavior.

EXPERIMENT I : Unilab Surgibone Inlay graft

This experiment showed that Surgibone granules were well tolerated and were rapidly incorporated into new bone when grafted in the tibia of rat. No foreign body reaction was observed around the granules allowing for incorporation of the granules in the new bone during the first week following implantation. New bone formation lead to a progressive bridge in between the granules and their incorporation appeared at its maximum during the four to seven weeks interval. During that time period, the thickness of the tibia was significantly increased when compared to control group.

Initially, the control group showed rapid filling of the defect by new woven bone. However, the thickness of bone observed in the first week was not maintained after this time interval. Remodeling of the cortical bone occured rapidly, such that, by the third week, the control leg demonstrated a normal appearance and thickness. In contrast, the grafted leg demonstrated a delayed remodeling process so that the maximum thickness was maintained up to eight weeks. Thereafter, the granules and the new bone appeared to be slowly resorbed.

At 32 weeks, the thickness of the cortical bone of the tibia of the grafted leg was more or less the same as the control leg. The granules were incorporated in the cortical layer which demonstrated a thickness comparable to that of the control. The granules appeared smaller at 32 weeks, and this observation was confirmed by morphometric analysis. These

results indicated that, at 32 weeks, the inlay granules appeared approximately two times smaller then those at week one. Similar results were observed by previous investigations using different types of bovine bone graft in which resorption was also significant (Maatz, 1957; Karges, 1963; Salama, 1983; Klinge,1992; Smiler, 1992).

It can be deduced from this experiment that the ideal time for titanium implant placement following bovine bone grafting in the rat model, would be during the four to seven weeks period. At this point in time, it is felt that the osseointegration of a titanium implant may occur because of the presence of sufficient bone thickness and new bone bridging the granules. However, it cannot be deduced if the thickness of bone will be maintained by placing a titanium implant in a tibia at that stage as there appeared to be a strong tendency to remodel. Thus, the rat tibia may not be the ideal model to investigate combination of bovine bone graft and titanium implant placement. Nevertheless, this animal model has provided some basic histological information on tissue response of the Surgibone granules and may be the simplest method of determining this product's response to titanium implants.

The clinical applications of bovine bone grafting for our human patients includes the maxillary sinus augmentation as previously mentionned. Surgibone granules appear to be an interesting alternative when used alone or in combination with autologous bone grafting.

In the case of an atrophic posterior maxillary ridge, a sinus lift procedure is sometimes required prior to placement of titanium implants (Boyne, 1980). To our knowledge only two case reports have been published with respect to sinus augmentation using bovine bone alone (Smiler, 1992) or in

combination with allogenic bone (Garey, 1991) prior to placement of titanium implants.

Even if we consider a sinus lift procedure as a three wall bony defect, the scarcity of cancellous bone in that region, with its lack of pluripotent cells and nutrient channels might compromise osteogenesis if bovine bone granules are implanted alone. As stated by Scoop (1966), a three wall bony defect has a great potential for osteogenesis but the vascularity and the presence of endosteum in tissue bed also influences osteogenesis.

If the granules are placed into a three wall defect, such as that for a sinus lift procedure, the contribution of the host, as described by the two phase theory of the bone healing, will be minimal. In fact, the thin cortical walls of the sinus offer a poor site to supplement the granules with host mesenchymal cells. The model utilized for this investigation created a three wall defect which was different as the granules were placed in a recipient site defect which contained abundant marrow elements and endosteum. This allowed for a rapid incorporation of the granules into new bone.

Since our ultimate concern was the application of Surgibone in conjunction with titanium implants, the use of these granules may still appear attractive for sinus augmentation if combined with osteogenic cells. Osteogenic potential of red marrow has been demonstrated by numerous investigors (Pfeiffer, 1948; Urist, 1952; Bloom, 1960; Burwell, 1964a; Friedenstein, 1973). Supplementing osteogenic cells to bovine bone to enhance osteogenesis is not a new concept (Gupta, 1982; Salama, 1978; Plenk, 1972). By mixing bovine bone granules with fresh autogenous marrow, one could very closely replicate the model that was utilized for this experiment. Thus, a combination bovine bone - autogenous

marrow graft might be successful for maxillary sinus augmentation. In fact, maxillary sinus augmentation in humans using a combination of hydroxyapatite and autologous bone has already been described (Moy, 1993) and preliminary results are promising. Since, porous hydroxyapatite is a relatively good osteoconductive material (Uchida, 1985), it can be hypothetized that bovine bone will behave in a similar manner, if not better than, hydroxyapatite. Moreover, the ability of Surgibone to resorb offers a distinct advantage over other alloplastics including hydroxyapatite. The resorption of the bovine bone granules will provide an increased surface contact of new bone to the metal implant.

By supplementing Surgibone with osteogenic cells one may argue that a second surgical procedure is still required to harvest the autologous bone. Consequently, one may initially question the value of bovine bone graft. However, the amount of osteogenic cells required to induce bone healing when combined with bovine bone was found to be comparatively small (Gupta, 1982). Therefore, a marrow aspiration or a cortico-cancellous block of bone harvested from the chin might provide sufficient osteogenic cells to supplement the bovine bone material. This will obviate the need for a more invasive iliac bone harvesting surgery. Marrow aspiration can be performed under local anesthesia. The chin graft is a very simple procedure which is located in the same surgical field as the augmentation procedure and, once again, may be performed using local anesthetic. Thus, supplementing osteogenic cells with bovine bone grafts appears to be a potentially better alternative for maxillary sinus augmentation.

In a clinical situation where the defect contains endosteum or cancellous bone and or marrow elements, it may be deduced that the Surgibone alone will perform very well as a bone defect filler. An example of such a defect might include

a cyst of the jaw that, following enucleation, may leave a walled defect and exposed cancellous bone. The size of the defect will also greately influence osteogenesis (Scoop, 1966). In fact, Hollman (1970) did not succeed when he attempted to fill a large osseous defect with kiel bone in the human jaw.

In summary, applications of bovine bone granules depends on several factors to be successful: the size of the defect, the presence of marrow or endosteum, and the general characteristics of the defect. These are all important factors to consider when selecting a bone graft material for a patient. Bovine bone in the inlay situation appears to be well incorporated and may be the material to consider in selected circumstances.

EXPERIMENT II : Unilab Surgibone Onlay graft

The tissue response during the first three weeks following implantation of the onlay graft was characterized by fibrous tissue invasion. However, at the lateral aspect of the graft, callus - like formation was observed. This appeared to mature over time until the inferior aspect of the graft became integrated with the tibia.

Incorporation of the onlay graft into new bone began at the inferior aspect of the graft. This process began three weeks following implantation and never progressed into the superior aspect of the graft. By sixteen weeks, the superior aspect of the onlay graft was encapsulated by fibrous tissue. The net result yielded, bone formation for approximately one quarter of the initial height of the onlay implant.

At 32 weeks, resorption of the graft was qualitatively appreciated by the thinning and the loss of continuity of the

implant trabeculae. This resulted in fragmentation of the implant. Measurement of bone thickness confirmed this observation indicating that the onlay graft appeared approximately 3.5 times smaller at 32 weeks when compared to the first week. Resorption of the bovine bone appeared to be more significant in the onlay graft when compared to the inlay. Although resorption and encapsulation were the striking features at 32 weeks, a significant residual increase in bone thickness was observed when compared to the control leg of the same time interval.

From this experiment, we can postulate that the onlay graft has osteoconductive potential but has no osteoinductive properties. Osteoinduction is the process in which mesenchymal cells from the recipient bed in contact with the grafted bone matrix will differentiate into bone forming cells (Huggins, 1970; Urist, 1976). In this experiment, bone incorporation of the graft has occured only at the inferior aspect. Bone forming cells probably originated from the host cortical bone, which cells were already differentiated to form bone. Thus, it can be hypothetized, that true osteoinduction was not seen at the inferior aspect of the graft. Furthermore, central and superior aspects of the onlay graft did not show any sign of osteoinductive potential as the existing fibroblasts never differentiated into bone forming cells in these areas.

One of the possible drawbacks of the model utilized for this experiment was the method of fixation of the onlay graft on the outer surface of the tibia. It consisted of a single circumtibial suture around the implant following which the muscles were closed to cover the implant. Micromovement may have created the picture that was seen. A fibrous union was initially observed at the interface between implant and host bone which was followed by callus formation. This may have resulted from this movement and may also be related to the

fragile method of fixation. Others have found similar problems when a graft is not adequately stabilized, as was stated by Taheri (1972), "Kiel bone which has no osteogenic inductive potency, must be placed in a vascular field and be well immobilized against a cancellous bone bed" to avoid fibrous union.

In order to improve osteoconduction and stabilization of the graft, a combined inlay-onlay graft model may facilitate migration of the mesenchymal cells from the medullary cavity via the inlay portion. Bone conduction may then proceed towards the onlay (outer) portion of the implant and yield improved bone retention. Uchida (1985) used a similar method to test the osteoconductivity potential of porous ceramic implanted in the tibia of rat. He found new bone formation only in the part of the ceramic intruding into the bone and none in the portion protruding (onlay) into the soft tissues outside the tibia. However, this may be more true for ceramics as these are less osteoconductive than bovine bone (Klinge, 1992).

An attempt was made in this experiment to simulate the clinical situation where an onlay graft, in the form of a block, is used to augment the alveolar height of an atrophic jaw. As was discussed earlier, previous investigations with onlay grafting using bovine bone have shown controversial results. Pinholt (1991) augmented alveolar ridges of rats with Bio-oss to find little or no bone formation and a foreign body reaction resulting in a fibrous nodule. Boyne's results (1987) were quite the opposite to those of Pinholt (1991). He successfully augmented the alveolar ridges of monkies with bovine bone (Bio-oss) and found that this material was successful and provided superior augmentation to that of hydroxyapatite. He also demonstrated that the particles were gradually replaced by the host bone during which time bone strength and structure were maintained.

The results of this investigation were found to be situated somewhere between these two studies. No foreign body reaction was observed and new bone slowly replaced the bovine bone at the inferior aspect of the onlay graft, which did result in increased thickness of the outer surface of the tibia. However, like Pinholt (1991), we found a fibrous encapsulation of the superior aspect of the implant. Thus, if one is to consider bovine bone grafting in a clinical situation, overcorrection would have to be built into the onlay grafting technique, in order to obtain the desired augmentation of bone prior to the placement of a titanium implant.

Another solution to improve the performance of this type of graft may be the utilization of a demineralized form of bovine bone. Guizzardi (1991) demonstrated a good osteoinductive potential with this type of graft. The demineralized form of bovine bone may release the BMP necessary to stimule the mesenchymal cells. As discussed earlier, in order to differentiate into bone forming cells, the host mesenchymal cells and or fibroblasts must be stimulated. However, the BMP in Surgibone may have been denaturated during processing, as postulated by Chappard (1991), therefore, supplementing it with exogenous BMP may induce bone formation and aid the incorporation of an onlay graft.

This concept was investigated by Ono (1991) using hydroxyapatite. He found that when hydroxyapatite was implanted subperiostealy in the rabbit skull only marginal bone formation was observed, whereas, when BMP was added, very active bone formation was found, not only on the interface in contact with the skull, but also surrounding the whole particles.

Once again, bovine bone offers the advantage of being gradually replaced by new bone which is not seen with hydroxyapatite. If supplementation of bovine bone with exogenous BMP can enhance osteoinduction, then the onlay graft may become an excellent method of augmentation prior to placement of a titanium implant, especially if combined using an inlay - onlay model.

CONCLUSION

It appears from the results of this investigation that bovine bone is rapidly incorporated into new bone especially with the inlay model. When grafted in a site containing abundant endosteal cells or marrow elements, the granules can be implanted alone with predictable result. If grafted in a recipient site denuded of cancellous bone, supplementation with marrow elements is prefereable to maximize bone formation.

When used as an onlay graft in the form of a block, the results were less predictable. Encapsulation by fibrous tissue persisted at the superior aspect and parts of the lateral aspects of the graft whereas the inferior aspect was fused to the host cortical bone. However, a resultant increase thickness of the bone was observed at the inferior aspect of the onlay graft. The inlay - onlay graft model has been proposed in order to improve stabilization, and subsequently, may enhance osteoconduction into the onlay portion of the graft.

Clinical application of bovine bone appears more favorable with the inlay model which can be related to a maxillary sinus augmentation procedure.

Future investigations using titanium implants in conjuction with the bovine bone grafts appears to be possible expecially with the inlay model.

APPENDIX

Techniques

This section provides a detailed description of the laboratory techniques used in the experiments.

1. PERFUSION:

Fixation of all animal specimens was performed using a 5% glutaraldehyde solution. To make a 5 % glutaraldehyde solution it was necessary to mix 10 ml of a 50 % solution in 100 ml of water and add 0.05 gm of 0.05 M Cacl2 (anhydrose) with 0.07 gm of 0.05 M NaCa codylate both in 100 ml of water. The Cacl2 and NaCa codylate were combined to bring the solution to 900 mls with water. The pH of the solution was checked to ensure a pH of 7.4 at 40^{o} C. Glutaraldehyde with water were then added to bring the solution to 1,000 ml.

In preparation for perfusion, one bag of Ringers lactate solution and one bag of the glutaraldehyde solution were hung and joined together with intravenous tubing leading to a stopcock. All air bubbles were removed from the lines and the level of fluid was brought right to the proximal end of the intravenous tubing.

Rats were anesthetized, placed on animal mounts and secured into position using elastic bands to ensure they did not move. The abdomen and thorax were swabbed with a 50 % alcohol solution. The flow of ringers solution was checked and adjusted so that the flow would break two inches from the needle. The bag was kept about five feet from the floor. The animal was cut open by lifting the skin overlying the abdomen and making an incision above the bladder. This incision was continued up to the xiphoid process, being careful not to pernetrate the diaphragm. The incision was then carried along

the inferior aspects of the ribcage bilaterally. Using blunt dissection the skin was reflected to the side.

Next the xiphoid process was held and lifted with a forceps while the diaphragm was cut up the sides at the junction of the diaphragm and the ribs. The diaphragm was then trimmed away and the xiphoid remained clamped up with its haemostat. This caused the lungs to collapse and the right atrium was then identified. While maintaining a firm grip on the heart, the needle attached to the intravenous tubing was inserted into the left ventricle. At the same time the right atrium was incised. The Ringers solution was then allowed to run approximately fifteen seconds.

When the liver blanched the Ringers soultion was disconnected and the glutaraldehyde was started. The flow was closely monitored as too fast a flow would result in solution spilling out of the animal's nose. Perfusion was continued for fifteen to twenty minutes after which the needle was removed and the tubing flushed with Ringers lactate. The animal's leg was dissected from its body and the tibia was removed. This was placed in glutaraldehyde for three hours, then washed in sodium cacodylate buffer two to three times at 4^{o} C, including an overnight change. The sample could have been stored indefinitely in cacodylate buffer at 4^{o} C. The sodium cacodylate washing buffer was prepared using the following formula: 4 % sucrose (osmolarity) - 40 gm\L, 0.05 M CaCl2 - 0.05 gm\L, and 0.1 M NaCacodylate - 21.4 gm\L with a pH of 7.4.

2. DECALCIFICATION:

The samples were tied in gauze bags and suspended in 1 L of 4.13 % disodium EDTA. The EDTA (ethylene diamine tetracetic acid) was made by combining 41.3 gm EDTA (4.13 % disodium) with 4.4 gm of NaOH in 1 L of distilled water. This

solution forms a buffering system at pH 7.0-7.4. Decalcification in this solution at 4° C with frequent agitation and change of fluid took on average 8 to 10 weeks. After demineralization the samples were washed with sodium cacodylate buffer. If this was not done a calcium\EDTA precipitate buffer will be left in the tissues. It was important to change the buffer frequently and rotate the tubes for 48 hours.

3. POST-FIXATION:

Reduced osmium was used for post-fixation. The tissue was placed in reduced osmium for two hours and only enough osmium was used to cover the tissue. The reduced osmium (1.5 % FeCn + 1 % osmium) was made by dissolving 0.6 gm of KFeCn (potassium ferroaxomide) in 30 ml of distilled water. Once this was dissolved, 10 ml of 4 % osmium was added. Following post-fixation the sample was washed in sodium cacodylate buffer for a few hours to a day at 4° C.

4. DEHYDRATION AND INFILTRATION:

The decalcified tissue was placed in graded acetones for 15 minutes each (30 %- 50 %- 70 %- 80 %- 90 %- 95 %- 100 %- 100 %- 100 % acetone) for dehydration.

The sample was then ready for Epon infiltration. The Epon mixture was created by combining 23 gm of Epon resin (812) with 14 gm of 98.1 % dodecenyl succinic anhydride (hardener) and 13 gm of 99.5 % Nadic methyl andride (hardener) and 1.0 ml of trimethylaminomethylphenol (accelerator). The following sequence was used:

1. 1:1 (Epon : Acetone) - 6 hrs. with rotation

2. 2:1 (Epon : Acetone) - overnight with rotation

3. 3:1 (Epon : Acetone) - 4 hrs. with rotation

4. 100 % Epon - 4 hrs. in a vacuum

The specimens including bone and the implant were embedded in modified Beam capsules and oriented so that the coverscrew was facing the side of the capsule. They were left to embed in the oven for 2-4 days at 60° C. It was important that all air bubbles be eliminated from the Epon and that the bubbles were not introduced into the mould or capsule by the pipette.

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