THE USE OF IRRADIATION TO STERILISE BONE ALLOGRAFTS

John Patrick Ivory MA MBBS FRCSEd FRCS(ORTH)

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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ABSTRACT

Large skeletal defects are often encountered in the surgery of bone tumours, revision arthroplasty and trauma. Skeletal stability can be restored using either large endoprostheses or structural allografts. The latter have the advantage of restoring bone in the defect. However, transplantation of allograft bone carries with it the risk of transmissal of contaminant or blood borne infection. Sterilisation of such grafts is commonly performed by the use of gamma irradiation.

If grafts are harvested in a sterile manner, there may not be the need to sterilise them for the purpose of removing bacterial contaminants. The first part of this study was the audit of an allograft bank to determine the incidence of contamination and the risk of subsequent infection. Five percent of femoral head grafts and eighteen percent of bulk grafts yielded positive bacterial cultures. The cultures indicate that the organisms which contaminate an individual graft are not the same organisms that cause subsequent clinical sepsis of that graft. The literature suggests that other factors, such as the size and type of operation in which the grafting is performed, may be of more importance than the contamination rate of the graft in the development of subsequent sepsis.

Blood borne disease may be eliminated by irradiation of bone graft at sufficient dosage. However large doses of irradiation may weaken the biomechanical properties of the graft or interfere with its incorporation. The second part of this study was the development of a simple animal model of allograft incorporation. The third part of

the study used this model to explore the question whether irradiation of bone grafts, at a commonly used dosage, interferes with graft incorporation. The results confirm that there is a marked difference between the incorporation of autograft and allograft. However, no difference was observed between the incorporation of irradiated and non-irradiated allografts.

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CHAPTER I
INTRODUCTION

Overview

Estimates suggest that over 150,000 bone graft transplants are performed each year in the United States of America (Tomford 1994). Excision of bone tumours may lead to large defects which require replacement with a bone graft of sufficient structural stability to replace the lost bone and restore mechanical function (Mankin 1983). Smaller defects in fracture non-unions and spinal fusions require bone graft, not necessarily for their structural properties but rather as a means of encouraging bone union. In revision joint replacement, large defects may require a larger structural bone graft which can provide mechanical support for the new hip prosthesis (Allan 1991). Smaller defects may be need to be filled with bone to encourage regeneration of bone around the new implant (Ghie 1993).

Bone grafting is a more attractive solution (Freidlander 1987) to bone defects than massive prosthetic replacement because of its ability to replace the lost tissue by a similar type, which integrates with the host. There is evidence that autograft (which is a bone transplant from the same individual) is the most suitable type of graft for encouraging union, incorporation into the host and bridging of the defect (Burchardt 1983, Stevenson 1992). However, the supply is limited and there is considerable donor site morbidity.

Allografts (which are transplants from different individuals of the same species) are available in larger sizes and any bone defect can be bridged by a corresponding structural graft. Xenograft bone (which is obtained from different species) potentially is in unlimited supply but the preparations do not incorporate as well and are unsuitable for bridging large defects because of structural failure. Bone substitutes, which include polymers such as polymethylmethacrylate and bioactive ceramics, are usually only suitable for smaller defects.

The use of allograft is not without complication. Bone grafts may fail to incorporate because of immunological rejection leading to resorption of the graft (Stevenson 1992). The bone graft may not unite to the host bone or may fracture because of mechanical overload (Berrey 1990, Thompson 1990). The use of bone graft may bring with it the risk of transmissal of blood or tissue borne infectious disease (Shutkin 1954, Eggin 1992, Simmons 1992). Infection of the graft may occur (Lord 1988, Dick 1994), particularly with larger grafts, often requiring removal of the bone graft concerned.

Factors that may contribute to infection include contamination of the bone graft during harvesting, storage or re-implantation. To reduce the risk of contamination and of transmissal of blood borne disease, bone grafts have been sterilised by methods which include heat, chemicals, ethylene oxide and irradiation. Of these, irradiation is the most widely used because of its convenience. There are concerns, however, about the effects of irradiation on the biomechanical properties of the graft and on the subsequent biological incorporation of the graft (Urist 1974, Pellet 1983).

This study examines extent of the clinical problem of sepsis and the use of irradiation as a method of sterilisation of bone grafts. The evidence for the detrimental effect of irradiation will be examined and an attempt will be made to model the clinical incorporation of irradiated large structural allografts.

History and Science of Bone allografts

The biblical story of the use of a rib from Adam to form Eve may be viewed as the first documentation of a bone graft (Genesis 2:21-22). The legend of Cosmos and Damian from around 1600AD alleges transplantation of a structural bone graft in which the leg of a man was amputated and replaced by the leg of a moor. The first documented bone transplant operation was performed by the Dutch surgeon Van Meekeren in 1668 on a patient in Moscow. He transplanted the calvarium of a dog to a cranial defect of a nobleman. Unfortunately, the Church disapproved of this combination and the transplant was removed (De Boer 1989).

MacEwen (1881) is credited with the first documented successful bone allograft. He collected the pieces of bone removed from six tibial osteotomies and inserted them into the 11cm defect in the humerus of a three year old boy. Two further operations were required to secure union. Judet (1908) performed a series of experiments transplanting osteoarticular allograft shells in rabbits. Lexer (1925) published the results of 300 human transplants of which 34 were fresh osteoarticular allografts. Although some centres have used osteochondral grafts in clinical situations with success (Mankin et al 1982, Gross et al 1983) it appears that the use of osteoarticular

grafts often leads to degenerative changes (Parrish 1973).

The end of the last century and early part of this century was an exciting time for research into bone osteogenesis and the incorporation of bone grafts. Albee established the first US bone bank in 1912 (Albee 1912), storing bone at 4-5 degrees centigrade. Albee believed that freezing would be harmful to the graft because it killed cells. Later experimental research by Bonfiglio (1962), Chalmers (1959) and Burwell (1963) established the advantages of freezing and other methods of preservation of allografts, which appeared to reduce the immune response to the prepared graft when compared to fresh allografts (Burwell 1994). These experiments then paved the way for clinical bone banks and transplantation programs. Bush (1947) and Wilson (1947) established the first two frozen bone banks. The US Navy Tissue Bank was established in 1952 using freeze drying for storage and irradiation for sterilisation. The early success of the bone banks and the ever increasing clinical indications for bone grafting led to many bone banks being established in the USA and the rest of the world (Burwell 1994). The development of the American Association of Tissue Banking (AATB) paved the way for setting standards and regulating processes in bone banks. This has been mirrored by the European Association of Musculo-Skeletal Transplantation (EAMST) and a Working Party of the British Orthopaedic Association.

With the emergence of bone grafting as a successful method of dealing with bone defects, associated theories arose to explain the mechanisms of incorporation. The theories proposed from research by the early researchers include:-

1. Specific osteogenic activity of the osteoblasts in the periosteum led to replacement of bone (Ollier, 1867).

2. All transplanted cells die and the dead bone is replaced by proliferation of new bone from the surrounding host bone (Barth 1893).

3. Both the periosteum and the endosteum of the graft are the source of new bone (Auxhausen 1908).

4. The bone graft cells grow and proliferate giving rise to new bone which is limited by the periosteum (Macewen 1912).

5. New bone is formed by metaplasia of the cells in the host connective tissue into which the bone graft is placed (Baschkirzew and Petrow 1912).

6. The old bone is gradually replaced by new bone (*creeping substitution*) produced by surviving cells in the periosteum, endosteum and superficial bone cortex according to the new functional demands imposed upon the bone graft (Phemister 1914).

Much of the early controversies centred around whether the cells in the transplanted bone survive and can therefore take part in the new bone formation. Urist and McLean (1952) showed that when fresh bone is transplanted into an ectopic site, new bone could be formed by cells that remained alive in the periosteum, cancellous bone and bone marrow. He described this ability to produce new bone as the *osteogenic*

potency of the transplanted tissue. However, bone was also formed by ingrowing cells of the host which were *induced* to form bone by contact with the substance of the transplanted bone. Gordon and Ham (1950) showed that osteocytes, in cancellous chips transplanted into muscle, died but that some osteogenic cells close to the surface of the chips survived, proliferated and gave rise to new bone. Further experiments demonstrated that freezing and thawing abolished this effect (Ham and Gordon 1952).

Predictable histological changes occur in response to bone grafting (Stevenson and Horowitz 1992). Bone graft, that is placed into a bone defect, is inserted into a milieu of fibrin, blood clot and inflammatory cells that are part of the normal response to injury of a bone. This haematoma is rich in platelet derived growth factors, other growth factors and cytokines. A local inflammatory response, characterised by the infiltrating lymphocytes, peaks between the second and third weeks. The host tissues respond to the injury by hyperplasia and proliferation (Rohde 1925) and there may be attempts by the host bone to deal with the defect in much the same manner as bone attempts to heal a fracture. Such response may be sufficient to heal the defect if it is small but the necessity for bone grafting often implies that it is not. A periosteal and endosteal response by the host is seen at the ends of the defect. However if the bone ends are widely separated in a large defect, no callus may be seen, as in amputation stumps.

Revascularisation of the injury milieu depends on the process of angiogenesis. Disrupted venules liberate endothelial cells which are attracted by chemotactic agents

and undergo proliferation (Folkman and Klagsbrun 1987). Numerous angiogenic factors have been studied with most interest being shown in fibroblast growth factor and transforming growth factor beta (Burwell 1994). The new vessel buds invade the cortical bone through the existing Volkmann or Haversian canals. Osteoclastic resorption of the graft occurs on the endosteal and periosteal surfaces, and at the junction with the host bone (Freidlander 1987).

As revascularisation proceeds, remodelling of the graft can begin. Adaptive remodelling to biomechanical stresses of loading can only take place when revascularisation has taken place and the graft is firmly united to the host bone. Successful incorporation of a graft can be defined as concurrent revascularisation and substitution without substantial loss of strength (Stevenson 1992).

New bone formed in response to fresh bone graft may be derived from osteogenic cells in the graft which survive the transplantation and are stimulated to undergo mitotic division. Experimental evidence suggests that such stimulation may be due to growth factors found in the bone matrix of the graft which include bone morphogenic protein, insulin like growth factors, transforming growth factor beta, fibroblast growth factors and platelet derived growth factors (Burwell 1994). Cells will only survive transplantation if the bone graft is fresh. Procedures such as freezing and freeze drying result in cell death and therefore other factors must be responsible for incorporation of these grafts (Ham and Gordon 1952).

New bone may also be produced by the surrounding connective tissue by the process

of osteoinduction, whereby one tissue causes a second undifferentiated tissue to differentiate into bone. In this inducing and reacting system, the reacting connective tissue cells need to have the potential to form osteoblasts. Such responsive connective tissue cells are known to exist in skeletal muscle, red marrow, subcutaneous tissues and other tissues such as the anterior chamber of the eye (Burwell 1994). Urist (1965) proposed the theory of autoinduction whereby the interaction of macrophages with dead bone produced inducing agents which induced primitive connective tissue cells to become dividing osteoprogenitor cells. These divided and matured into differentiated osteoblasts which formed new bone.

The characterisation of such an inducing agent, the bone morphogenic protein (BMP), proposed by Urist, was difficult because of its tight association with collagen. In 1979 Urist isolated an osteoinductive hydrophobic, low molecular weight protein from insoluble bone matrix protein. The amino acid sequence of BMP was characterised by Wozney et al (1988) who demonstrated different polypeptide fractions each of which could produce cartilage in vivo. The genes for BMP1, BMP2-A and BMP3 have since been mapped on the human genome (Tabas et al 1991) and recombinant BMP-2 has been shown to produce new bone in the rat (Lane 1992). BMP2-A and BMP3 are members of the transforming growth factor beta family. Other factors thought to be important in bone induction are platelet derived growth factor, fibroblast growth factor, macrophage derived growth factor and interleukin-1 (Burwell 1994).

Other work suggested that there may be a species difference with regard to

osteoinduction, with higher species, such as canines, evoking less osteoinduction than in the rat (Schwarz et al 1989). Urist (1989) and Burwell (1994) believe that the bone marrow stromal cells may be important in primates. Bone marrow contains determined osteogenic precursor cells and inducible osteogenic precursor cells.

Phemister (1914) drew attention to the *creeping substitution* of old dead graft bone by new live bone. This process, often termed *osteoconduction*, depends on osteoclastic, osteogenic and angiogenic cells. Enneking et al (1975) examined the remodelling of bulk fibular bone grafts inserted subperiosteally in dogs. The cortical bone weakened due to increase in the internal porosity at six weeks and remained so until six months. The strength returned to near normal by one year. Further remodelling may take place according to the functional demands placed upon the bone, according to Wolff's law.

The incorporation of bone graft can be summarised thus (Urist 1980):-

1. (Minutes to hours) Inflammation and proliferation of cells in the graft bed.
2. (One to seven days) Osteoinductive response of cells in the graft bed to BMP in the bone graft.
3. (Months to years) Osteoconduction consisting of revascularisation and new bone formation.
4. (Two to twenty years) Mechanical function with remodelling.

Although allografts can incorporate and answer many of the problems of large defects in bone there are a number of difficulties to be overcome.

Some problems with allografts

1. Rejection.

Implantation of allograft excites an immunological response in the recipient. The clinical superiority of autograft over allograft is well documented and depends on the recognition of self and non-self (Burchardt 1983 and Stevenson 1992). Bone grafts bear Class I and Class II antigens which are products of the genes located on the Major Histocompatibility Complex. Class I antigens are found on the surface membranes of virtually all nucleated cells including osteocytes. Class II antigens are found on B lymphocytes, certain macrophages and other antigen presenting cells including bone marrow cells.

In the normal process of rejection of a parenchymal organ like the kidney, cell mediated and antibody mediated cytotoxic processes, specific for class I and II donor antigens develop after transplantation. Such processes have an effect on the vasculature of the organ and result in cytotoxicity of the parenchymal cells. However, frozen allograft bone contains no living cells and does not contain a vascular tree. Experimental studies have shown both cell mediated and antibody mediated responses do take place in frozen allograft bone, but the mechanism of presentation of the antigen and the precise target for the antigen specific response is unclear (Freidlander 1976). The clinical success of frozen allografts may be related to disruption of cell membranes and therefore the presentation of surface antigens. It may also lead to activation of antigen specific suppressor T cells which can modulate any rejection process (Stevenson 1992). One helpful study looked at correlation between failure of the allograft in 26 large allografts in tumour patients and tissue typing with the major

histocompatibility complex (Musculo et al 1987). Twenty three patients had biopsies of their grafts which were assessed blinded to the tissue typing results. There was no correlation between failure and tissue incompatibility but two grafts showed round cell and vascular invasion suggestive of an immune response to donor antigen. The authors concluded that there are many variables leading to host/graft response including methods of fixation of the graft and polymorphism of the HLA system. Lord et al (1988) found that non union of the graft was more common in patients whose grafts got infected and suggested, without supporting data, that infection may be a means of presentation of graft rejection.

2. Transmissal of disease

Transplantation of foreign material into a different individual, brings with it the attendant risks of transferring a blood or tissue borne disease. Transmission of hepatitis B (Shutkin 1954) and hepatitis C (Eggen and Nordbo 1992) have both been reported following transplantation of frozen bone allograft. Transmission of the Human Immunodeficiency Virus (HIV) has been reported from untested donors (Centre for disease Control 1988) and from antibody negative donors (Simonds et al 1992). The latter report highlighted the problem of infecting multiple recipients from a single donor. With proper screening, it has been estimated that the risk of HIV transmission is less than one in a million but if proper procedures are not followed this may rise to one in 161 (Buck et al 1989). However, one bone bank in Paris has reported that 10% of their cadaveric donors were HIV positive (Hernigou et al 1992). There are also theoretical concerns about transmissal of other diseases, such as arthritis and malignancy, which do not have clear aetiologies. Creutzfeldt-Jakob

disease is a rare slow-virus disease, which has a latent period between infection and symptoms often in the region of 20 years. It can be transmitted by tissue transplantation but has yet to be recorded in musculoskeletal transplantation. Currently, there is no serological test for this and therefore screening must exclude those donors with dementia or anyone who has received pituitary derived hormones.

The American Association of Tissue Banks (1992) and more recently the European Association of Musculoskeletal Transplantation (1994) have drawn up guidelines for the screening of donors to reduce the risk of transferring a blood or tissue borne disease with the graft. In general the same principles apply for living and non-living donors with a few exceptions. Individuals are screened by direct questioning of the donor or the donor's relatives in the case of cadaveric transplants. Criteria for rejection, as suitable donors, include infectious disease including tuberculosis, malignant disease, central neurological degenerative disease including dementia, autoimmune disease and history of having been treated with pituitary derived hormone. The criteria for exclusion, because of high risk of HIV infection, include homosexuals, drug abusers, haemophiliacs, prostitutes or their contacts and people from Haiti or Central Africa (EAMST 1994).

Donors are also screened by serology tests for HIV (human immunodeficiency virus), HTLV-1, Hepatitis B, Hepatitis C and syphilis. Living donors, such as those donating their femoral heads whilst undergoing hip replacment, should be retested for HIV, hepatitis C virus and hepatitis B surface antigen at least six months after retrieval of the grafts because, if the graft is retrieved in the time window between infection and

seroconversion, a false negative result may occur. In the case of non-living donors, such as those involved in fatal road traffic accidents, from whom the recipients receive such organs as kidneys, lungs etc, there is the possibility of serological testing the recipient six months after the organ donation. However this may raise some ethical problems and it is recommended in non-living donor cases, that the donor has the polymerase chain reaction (PCR) test performed, a test for the antigen of HIV. The PCR test can also be used on living donors to replace the six month retest but, due to its high cost, it is not routinely used. Another development has been the HIV-1 p24 antigen test which is based upon detection of parts of the virus itself and may be another useful addition to the screening tests. EAMST recommend testing of Hepatitis core antibody in addition to hepatitis B surface antigen because in a small number of cases the surface antigen may be negative although the core antigen DNA will still be detectable, especially by PCR (EAMST 1994).

3. Infection

Bacterial infection following implantation of a bone allograft is a serious complication often leading removal of the graft (Dick and Strauch 1994) and even amputation (Lord 1988). The rates of infection vary in different series according to the type of grafts used and the indications. Small allografts used in revision hip replacement have been reported as having a negligible infection rate. Tomford (1990) did a postal questionnaire to surgeons who had used their grafts in a two year period. They had a 97% response to the questionnaire, at an eight to thirty-two month follow-up and none of 113 small bone grafts became infected. Hart (1986) had no infections after

the use femoral head allografts in 101 patients.

Larger proximal femoral allografts used in revision hip arthroplasty have been associated with infection rates ranging from 0% in twenty-two patients who received a large freeze-dried graft (Head 1987), 4% in fifty-one frozen allografts (Tomford et al 1990) to 6.8% in forty-four patients who received large frozen allografts (Allan 1991 and Gross 1985). Mankin's early series of large allografts used in tumour resection had an infection rate of 14% (Mankin 1983 and 1987). A more detailed later retrospective review of 283 grafts followed up for two years or more showed an infection rate of 11.7% (Lord 1988). Multiple regression analysis in this series suggested that those with more extensive surgery leading to more loss of bone, soft tissue or skin and those with multiple operations were particularly at risk. Patients who require adjuvant chemotherapy or radiotherapy have an infection rate of 11.1% (3 of 27 grafts) (Dick et al 1985) in comparison to 4.6% (3 of 65 grafts) in a group without adjuvant therapy from the same centre (Mnaymneh 1985). The two groups also differed in that Dick's series was both freeze-dried and frozen grafts whereas Mnaymmneh's series was only freeze-dried. Skin necrosis was present in two of the three infections in Dick's series and may be a significant precipitant of infection. Lord et al (1988) found that skin slough was an associated predisposing factor in 13 of 33 patients with infected allografts.

Gram positive organisms are the most common infecting organisms and the mean time to recognition of the infection is 4.3 months (Lord 1988). In only one case out of 33 infected massive allografts, in Lord's report, was contamination of the graft the

obvious source of the infection. However, Tomford (1990) reported that seven of thirteen infections in massive allografts may have been attributable to the allograft and was a definite possibility in three. Post-operative infection may be caused by the introduction of organisms in one of three phases: the pre-operative, intra-operative or post-operative periods (Dick and Strauch 1994).

Bacterial contamination of massive allografts at procurement has been reported to vary between 3 and 40% (Veen 1994). The Massachusetts General Bone Bank reported a contamination rate of 28 out of 207 massive allografts and 11 of 93 femoral heads (Tomford 1990). Tomford separated out a group of seven infected grafts in which there was no satisfactory other explanation for infection such as skin necrosis or chemotherapy and he regarded infection in this group as being possibly due to the graft itself. However, the infecting organisms in this group were composed mainly of *Serratia*, Enterococcus and *Ent. Cloacae* unlike the predominant gram positive organisms which contaminated the whole group at procurement. Tomford compared their infection rate with that of reports of sterilised endoprosthetic replacement of skeletal defects and concluded that clinical infection may be due to factors other than bacteriological contamination. Gristina and Costerton (1985) studied routine and extended microbiological cultures from 10 infected orthopaedic implants and compared these with scanning and transmission electron microscopy of the samples. The routine and extended cultures were different in five cases. The electron microscopy showed evidence of a thick biofilm. The authors postulated that routine cultures may not be sensitive enough to disturb enough of the bacteria from the biofilm to ensure full detection especially if the infection is polymicrobial.

Veen (1994) has shown that a contamination rate of 54.9% can be found if careful subculture methods are used but that this will drop to 23.1% if routine cultures only are used. If the bone itself is cultured, a contamination rate of 92% can be found even when a meticulous aseptic procurement procedure is followed. Over 90% of contaminating organisms were skin commensals (*Staphylococcus aureus, Corynebacterium species and Propionibacterium acnes*). He compared the contamination rates in cases where three to four people were responsible for procurement with those where five to six people did the retrieval. He showed a statistical difference in favour of a reduced number of operating room personnel (3-4 people: 166/386(43%) versus 5-6 people: 305/471(65%) $p < 0.0001$). His view is that all, or nearly all, allografts are contaminated because of the number of people involved in the operating room during procurement.

Reviewing their clinical infection rate of 2 out of 82 large allografts he concludes, like Tomford (1990), that the graft probably had little role to play in the infection. Both infected grafts were from donors who provided other grafts which were transplanted without infection. One graft was infected with *Klebsiella* which was not one of the contaminating organisms in this series. Veen surmises that although grafts are probably contaminated in most cases, this represents an acceptable bioburden to the recipient. Of more importance for infection, is the length and type of operative procedure for which the grafting is being performed as these affect the intra-operative contamination of the graft. This does not negate the use of routine bacteriological screening methods which exclude those heavily contaminated grafts which might

exceed this "acceptable" burden.

Postoperative contamination may be because of local invasion by bacteria in wound haematomas or skin necrosis, seeding by bacteraemias or because of impaired immune responses by the recipient (Dick 1994). Lord et al (1988) found that skin slough was the most common identifiable cause of infection, especially as poor wound healing was typical in these tumour cases because of chemotherapy and local radiotherapy.

The use of irradiation

Some bone banks employ sterilisation methods as a routine for all of their bone grafts. Other banks are more selective, performing sterilisation of bone allografts when the cultures, taken at the time of procurement, show evidence of bacterial contamination. Some processing procedures for storage of bone graft e.g. freeze drying require a secondary sterilisation procedure to ensure sterility. The methods commonly used are gamma irradiation, ethylene oxide, chemical sterilisation and heat. Gamma irradiation is the most frequently used method followed by ethylene oxide. Phillips (1994) reported that the advantage of irradiation is that no significant temperature, physical or chemical changes are induced which could interfere with the function of the tissue. The high penetration of irradiation enables the bulk graft to be sterilised in its packaged form. The process is precise and when applied accurately, can achieve sterility. Despite Phillips' (1994) enthusiasm for this method of sterilisation there are reports of the possible deleterious effect of irradiation on bone graft function and these will be discussed below.

The first reports by Turner, Cohen and Devries of the use of irradiation to sterilise bone allografts were in the 1950s (See DeVries 1958). The bactericidal effect was thought to be due to the direct effect of the irradiation on the bacterial nucleoprotein and a secondary effect due to free radical formation. Current theories suggest that ionising irradiation strips electrons from the atoms of the material through which the irradiation passes and this initiates a chain of reactions. Ionisations occur principally in water resulting in the formation of hydroxyl and proton highly reactive radicals. Single strand and sometimes double strand breakage of DNA ensues. The structural defects in the microbial DNA cause errors in protein synthesis leading to cell death (Russell 1990).

The electron spin resonance studies of Stachowicz et al 1970 have shown that free radicals are liberated by both the collagenous and apatite parts of bone. Further work has shown that peptide bonds are cleaved by irradiation (Buring 1970) and that the cross linkage of fibrillar collagen is destroyed causing obliteration of cross banding of the collagen (Buring and Urist 1967, Weintroub and Reddi 1988). The result is that irradiation may increase the solubility of the collagenous part of bone matrix.

Turner (1956) summarised the experiments of Moriarty to determine the sterilising dose of radiation for pure cultures of a wide range of micro-organisms. The most resistant were noted to be the spore forming organisms and in particular Bacillus subtilis which was the reference used at the time for radiation sterilisation. Moriarty found that a dose of 2.5 Mrads (20 Kgray) was required to sterilise this organism. In a series of experiments, Turner et al (1956) demonstrated that frozen and freeze dried

bone subjected to a radiation dose of 1.0 Mrads (10 Kgray) caused complete sterilisation of an inoculum of staphylococcus aureus. DeVries et al (1958) determined the bactericidal levels of irradiation in dogs inoculated with pure cultures of Clostridium botulinum and Clostridia tetani or mixed cultures of various gram positive and gram negative rods. Like Moriarty he found that a dose of 2 Mrads (20 Kgray) was the minimum bactericidal level. Other experimental studies have shown that irradiation to a dose of 2.5 Mrads provides greater than a 6 log reduction of bacteria in specimens of cortical bone (Bright et al 1983).

DeVries (1958) summarised the work of Jordan on virucidal doses of radiation in the rat tibia known to infected with viable viruses including vaccinia and poliomyelitis. Viruses were more resistant to radiation than non-sporulating bacteria. However they are more sensitive than bacterial spores (Morris 1972). A review of the available evidence by Wright and Trump (1970) suggested that a minimum dose of 2.4 Mrads would be adequate to inactivate high concentrations of almost all of the blood borne viruses studied.

Spire (1985) studied the effect of gamma radiation on the HIV virus. At doses of 0.25 Mrad viral samples were no longer infectious although the viral reverse transcriptase enzyme itself was unaffected by 0.75 Mrad. Inactivation of viruses is based upon the sensitivity of their nucleic acid to irradiation whereas their proteins are relatively resistant. It would thus appear that even a modest dose for bacterial sterilisation will inactivate the HIV virus. Hernigou et al (1992) reported inactivation of HIV in bone by 2.5 Mrads (25 Kgray) of irradiation. Conway et al (1991) showed that the amount

of irradiation required to reduce the viral load by one log10 unit was in excess of 0.4 Mrad (4 Kgray). Irradiation sterilisation attempts to reduce the number of live pathogens to the generally accepted figure of 10^{-6}, which represents a probability of 1 in a million that a live organism exists after sterilisation. According to Conway (1991), assuming a bioburden of 1000 TCID 50 (concentration that will infect 50% of cells in culture "tissue culture infective dose") which has been shown in the blood of HIV infected persons, then 3.6 Mrad would be required to completely inactivate the HIV-1 virus. Conway et al (1991) recommended that donor screening and quarantine of grafts from live donors should be performed, since doses of 3.6 Mrad, required to guarantee sterilisation, may have harmful effects on the biological and mechanical properties of bone grafts. Indeed, work by Fideler et al (1993) supports this assertion that the most commonly used dose of irradiation (2.5 Mrads) may not be sufficient to guarantee sterilisation. HIV infected patellar tendon allografts irradiated at 2.5 Mrads were not uniformly sterilised on polymerase chain reaction testing. However it is possible for the PCR test to detect virus antigen, although the virus itself may have been destroyed and is no longer infectious.

Thus, it appears from the evidence that 2.5 Mrads of irradiation is sufficient to eliminate most bacteria and spores. The International Atomic Energy Agency (Van Winkle et al 1976) adopted 2.5 Mrads as the standard dose for sterilisation of medical products. Most viruses also appear to be eliminated at this dosage but there remains some doubt as to whether this dose is sufficient to eliminate the HIV virus from bone. Some authors state that the standard dose for irradiation of tissues should be increased from 2.5 to near 4 Mrads. (Conway 1991, Rasmussen 1994).

Biomechanical effects of irradiation

Kommender (1976) studied the effects of different modes of preservation of bone on the biomechanical properties of fresh human cadaver femurs. Machined samples of cortical femora were prepared by different methods and tested in compression, bending and torsion. Compression was done on cylinders of 8mm length and 5mm diameter, bending was done on rectangular blocks measuring 35 x 4 x 4 mm and torsion was done on cylinders measuring 15 x 4 mm. Each sample size was six or seven. Deep freezing to -78°C reduced bending, compression and torsion strength to 90-95% of that of intact fresh cortical bone (not significant). Irradiation of fresh bone by 6 Mrads did significantly diminish bending strength by 30%, compression by 20% and torsion strength by 35%. Doses of 3 Mrads or less did not significantly alter the biomechanical properties. Since these specimens have been prepared from only three pairs of femurs there is a strong possibility that the area where the sample has been taken from could influence the performance of the graft. Kommender reports that in a previous experiment (unpublished) he showed that the mechanical properties of samples taken from different parts of the femur showed no significant difference. He therefore randomly allocated different samples from each area of the femur into each group subsequently tested. This assertion that the samples from different parts of the femur behave the same way seems unlikely and other authors (Anderson et al 1992) have taken the trouble to match pairs of samples to overcome this problem.

Bright and Burstein (1978) performed a series of tests on specimens of cadaver femurs. Blocks were prepared and subjected to tension and compression forces. In specimens that were irradiated with 2.5 Mrad, the only biomechanical property

altered was that of the plastic modulus which was elevated in comparison to non-irradiated controls. In specimens that were both irradiated and freeze dried, the yield stress was reduced, the ultimate strain decreased and the plastic modulus increased. No dimensions of the blocks tested were given and only the result rather than the data was given.

Triantafyllou et al (1975) tested three point bending in samples of fresh adult bovine tibia machined to 48 beams of 8 x 0.5 x 0.5 cm from the long axis of the diaphysis. These were frozen and the trial groups were subject to freeze drying or irradiated to 3.0 to 4.0 Mrads or both. Irradiation reduced strength to between 50% and 75% of controls and the combination of irradiation and freeze drying reduced strength to between 10% and 30% of controls. No statistical comparison was made between the groups. Pelker et al (1983) concluded from a review of the studies of Kommender(1976), Bright and Burstein (1978) and Triantafyllou (1973) that below 3 Mrads there is little change in the strength of tested samples of cortical bone but above this there is a significant drop in the strength of bone which is magnified by combining freeze drying and irradiation.

Two studies suggest that there is no harmful effect of irradiation at 2.5 Mrads on cancellous bone. Anderson et al (1992) evaluated the compression strength of cancellous bone in 1 x 1 x 2 cm blocks cut from human proximal tibiae subjected to varying doses of irradiation. There were six to ten samples in each group. Irradiated and control samples were prepared from matching areas of the left and right tibiae to control for anatomical variations in bone strength. The compressive failure stress and

elastic modulus decreased significantly when the bone was irradiated at 6 Mrads. At doses less than this there was no significant decrease. Zhang et al (1994) examined the compressive qualities of freeze dried human iliac crest cancellous bone prepared in tricortical wedges. This graft was harvested in the manner in which it would be used in clinical practice. They could show no significant difference in the 81 specimens irradiated at 2.5 Mrads and the 22 non-irradiated controls.

A series of reports (Gibbons et al 1991, Haut 1990 and Rasmussen 1994) suggest that patella bone tendon bone allografts are significantly weakened in tension with respect to failure by irradiation doses ranging from 2 to 4 Mrads. All failures occurred in the midsubstance of the tendon rather than through bone.

In summary the evidence from the previous studies suggests that at 2.5 Mrad there is little harmful effect of the irradiation on the biomechanical properties of cortical and cancellous bone. However, there may well be harmful effects at doses above 3 Mrads. The previous studies looked at rectangular or cylindrical strips rather than whole segments of bone. In the clinical situation, cortical strips are sometimes used in revision hip arthroplasty but in the majority of tumour cases whole segments of bone are transplanted. None of the previous studies have attempted matched paired comparison of large segments of bone in the mechanical tests. Zhang's study (1994) is the only one to use graft samples in the manner in which they would be used in clinical practice. Most of the studies have relied on small sample sixes of six to ten specimens with a strong possibility that sampling errors and regional differences in mechanical properties may have influenced the result. Anderson's study (1992) is the

only to control for regional variation of the samples.

Of concern is the possibility, already mentioned, that the accepted figure of 2.5 Mrads may not be sufficient to eliminate all of the HIV virus and that the higher doses which might be required may have harmful biomechanical. It also appears that even relatively low doses of irradiation are harmful to patellar tendon grafts.

Immunological effects of irradiation

Implantation of fresh allograft excites an immunological response in the recipient. Different measures of this response have been studied by various workers including inhibition of new bone formation (Chalmers 1959, Brooks 1963), altered patterns of second set skin graft rejection (Chalmers 1959), changes in the regional lymph nodes draining the site of the graft replacement (Burwell 1963) and serological attempts to identify antibodies against bone extracts (Brooks 1963). Immunological intolerance results in poor incorporation of the graft. Reducing the immunogenicity of the graft may help prevent rejection of the graft, which will incorporate more readily. Various studies have demonstrated that the freezing process reduces the immunogenicity of the bone (Brooks et al 1963, Klinman 1981, Ostrowski 1969). Some studies have examined whether irradiation may help reduce the immunogenicity of the graft and these are described below.

Brooks (1963) looked at cell mediated immunity using the skin rejection model of Chalmers (1959). Frozen (-40°C), irradiated (2Mrad) allograft was transplanted into

a heterotopic site in mice. No definite effect was observed and Brooks felt that this may have been related to problems with the model used and the small sample sizes.

Ostrowoski (1969) showed a reduction in the lymph node blast cell reaction in mice to allogenic cancellous bone which was irradiated alone, frozen alone or irradiated and frozen in comparison to fresh controls. There appeared to be little difference between the three groups suggestive that the effects of irradiation and freezing are not additive.

Klinman (1981) investigated the immunological consequences of 2 doses of irradiation in mice (650 or 1,200 rads). The bone graft in these experiments consisted of a short segment of tibia transplanted either into a mouse of the same strain (syngeneic) or into another strain (allograft) and was internally fixed with an intramedullary pin. The immune response was measured by the antibody response using a complement dependant lysis assay. The recipients of allogenic irradiated bone responded slightly (650 rads) or not at all (1200 rads) compared to the strong antibody response to non irradiated allografts. Although not clear in the text of this paper, the grafts were probably fresh rather than frozen. Klinman concluded that irradiation appears to reduce the immunogenicity of the graft. Pellet et al (1983) showed reduced antibody development at very low doses of irradiation (10 Krad) compared to controls and antibody production was abolished at 100 Krad. It thus appears that irradiation does provide a protective effect from rejection but it appears unlikely that this is any more pronounced than the effect of freezing alone. The reduced immunogenicity may result in better graft incorporation.

Effect of irradiation on incorporation of the graft

In his study Klinman (1981) also investigated the consequences of 2 doses of irradiation in mice (650 or 1,200 Rads) on the healing of segments of mouse tibia transplanted either into a mouse of the same strain (syngeneic) or into another strain (allograft). Graft healing was assessed by torsion testing and the incidence of non union. Syngeneic grafts, whether irradiated or not, had a lower incidence of non-union (both 1 out of 9) than fresh or irradiated allografts (5/9 and 4/9 respectively). The grafts were probably fresh but this is again not clear in the paper. Klinman had shown in the previous experiment (*vide supra*) that irradiation of fresh allogenic bone reduced or abolished the immunological response. It would thus appear that despite reducing the immunological response to bone by irradiation, the incidence of non-union is still high in allogenic bone. No figures were given for torque testing in these groups. The tensile strength of the graft union was tested in an Instron machine and the results were expressed as a fraction of the intact opposite tibia. Irradiation of syngeneic grafts reduced the tensile strength of the union to that of the allograft group. Irradiation of allografts had no net effect; both irradiated and non irradiated allografts grafts healed poorly. Therefore, although irradiation appeared to reduce the immunogenicity of the allograft, it did not improve graft healing, but neither did it make it worse. This suggests that these measures of graft healing (percentage non-union, torque and tensile strength) are poor indicators of the immunological response.

Turner (1956) looked at the healing of cortical inlay grafts in both radii of thirty eight dogs. The grafts were either frozen or freeze dried and then subjected to doses of

irradiation from zero to six Mrads. Histological scores were given to assess healing. Although these measures are subjective the slides were evaluated by three different observers whose opinions were not grossly different. The results indicated that doses of radiation below 2 Mrads did not impair healing of freeze dried grafts but above 2 Mrads there was evidence of impaired healing. Frozen bone subjected to irradiation at 0.5 to 4 Mrads showed no impairment or slight improvement in healing. At 6 Mrads there was evidence of enhancement of healing in mild to moderate degrees.

Heiple (1963) studied the healing of cancellous allografts transplanted to the dog ulna using a histological scoring system. Grafts were frozen at -40 degrees and / or irradiated with 2 Mrads. The animals were sacrificed at 1, 2, 3 and 4 weeks; 3, 6 and 12 months. Frozen allografts were similar to controls until three weeks when they lagged behind autografts in the repair and replacement of the marrow, spongiosa and cortical bone. Union was also only slightly retarded and the end result at 1 year was the same. Frozen, irradiated grafts were little different to the autograft controls at one week, but thereafter showed definite evidence of delay in healing. However, at three months there was very little difference and at one year the results were much the same. There were only two to three grafts at each time point in each group and no statistical comparison was made.

Pellet et al (1983) used a cortical defect in the rat femur to study the effect of freezing and irradiation on the healing of allografts. Radiographs and histology were used to study healing but the details were not given. There were seven grafts in each

group. Non-irradiated frozen grafts appeared to lie inertly in the defect and the majority did not unite to the host. Irradiation at doses up to 1 Mrad showed similar healing rates to control fresh autograft but doses of 5 Mrads and above impaired healing. There were no grafts given doses of irradiation between 1 and 5 Mrads, the most interesting range to study.

Urist and his colleagues (1965, 1967) developed a model using decalcified bone matrix transplanted into an ectopic site to measure the amount of new bone formed. Urist (1974) believed that proteins in the bone matrix are responsible for the "osteo-induction" and that for full expression of this induction the bone has to be decalcified. In early experiments (Buring and Urist 1967) demineralised bone matrix from rats and rabbits was subjected to doses of irradiation up to 37 Mrads. Other samples of bone were irradiated prior to decalcification. A total of 656 samples were implanted in the muscle bellies of the anterior abdominal wall of 95 adult rats and 8 adult rabbits. The amount of new bone was estimated by direct measurement on radiographs. In rats, new bone was never seen in implants that received more than 1.5 Mrads but was seen at doses below this level and in non-irradiated controls. In rabbits, irradiation below 2 Mrads reduced the amount of new bone by 50% when compared to non-irradiated controls. At doses above 2 Mrads there was no new bone formed. A later report by Buring (1970) with increased numbers of rabbits showed that irradiation up to 0.5 Mrad had no measurable effect on new bone formation by demineralised lypholysed bone matrix. Bone matrix irradiated between 1.0 and 1.5 Mrads showed decreased new bone formation and irradiation of 2Mrad or above abolished new bone formation.

In later experiments, Urist (1974) transplanted decalcified irradiated bone allograft into an ectopic site in the abdominal wall of the rat. He varied the order in which irradiation and demineralisation were performed. New bone formation was assessed at 3, 4 and 6 weeks by x-rays, histology and dry ash weight per gram of implanted demineralised matrix. The results showed that samples irradiated prior to demineralisation produced less new bone than those demineralised before irradiation. Even bone that was demineralised prior to irradiation resulted in yield of new bone that was reduced by 60% at 2.0 to 3.5 Mrads and reduced by 90 % at 4 to 5 Mrads. Insoluble bone gelatin was the least sensitive preparation of bone to irradiation, with only a 20% reduction at 4 Mrads. In the same series of experiments Urist (1974) also transplanted controls of undemineralised irradiated bone and measured the amount of new bone formation by counting the number of new bone deposits and recording their location within 100 intercepts of a light microscope eyepiece grid. By this measure he found that bone subjected to doses of radiation less than 1.0 Mrads evoked small well developed colonies of new cartilage and bone within the interior of old vascular channels. At 2.0 Mrads this new bone was not seen. The bone degradative enzyme systems were assayed after irradiation and it was found that radiation had no appreciable effect on bone degradative enzymes.

Urist concludes that radiation reduces the capacity of allograft to induce new bone and also leads to the preservation of the harmful degradative enzymes. This reduction in induction appears to be most pronounced when bone is irradiated prior to demineralisation and least when the bone has been reduced to gelatin prior to irradiation. Urist explains this by proposing that the increased density of the normally

mineralised bone leads to an increased absorption of the radiation causing enhanced destructive effect.

Tetracycline labelling, microradiography, radiodensity, histological and radiological fusion, revascularisation and remodelling were methods of assessment used by Prolo (1982) to assess healing of circular defects in canine calvaria with differently prepared implants. Lypholized irradiated grafts (2.5 Mrads) produced significantly less new bone at six months when compared to lypholisation alone. However, this was only found on random point analysis of the projected images of the microradiographs onto a grid and all the other criteria of healing showed no difference between the two groups.

There are studies which provide contradictory evidence to that of Urist and his colleagues. Weintoub and Reddi (1988) reported the results of ectopically transplanted rat demineralised bone matrix which had been irradiated (1 to 15 Mrads). Bone formation was assessed by measuring alkaline phosphatase activity and by radioactive calcium incorporation. Irradiation at 2.5 Mrads was found not to destroy the induction properties of demineralised bone matrix. At 3 to 5 Mrads, there was even more mature bone than non-irradiated controls. At doses above 5 Mrads, the response was delayed. Schwarz et al (1988 and 1989) transplanted demineralised bone matrix that was irradiated with either 2.5 or 5.0 Mrads into either an ectopic or orthotopic (calvarial) site in rats. New bone was assessed by histology, measurement of alkaline phosphatase and histomorphometry. The results show that, at 5.0 Mrads, the osteoinductive response is destroyed. However, at 2.5 Mrads, the alkaline

phosphatase, histology and histomorphometry were the same as a control group but the mean net weight was significantly less in irradiated explants. In further experiments (Katz et al 1990), the insoluble collagenous bone matrix was prepared from rat diaphyses. Used alone, this preparation had no osteoinductive capacity but when recombined with osteogenin, osteoinduction was restored. Irradiation of the collagenous bone matrix did not have a deleterious effect on the new bone produced when osteogenin was recombined with it.

Clinical experience with allografts

There are now several series of long term follow-up of patients who have received massive allografts. They are associated with a high complication rate of infection, fracture and non-union. These complications often necessitate further operative procedures and carry considerable morbidity (Vander Griend 1994).

Fracture of the allograft tends to occur any time after the first six months, with an average time to fracture of 29 months and is rare after 4 years. It may be associated with non-union of the host graft junction (Berrey et al 1990) but other authors have not confirmed this (Thompson et al 1993). Mankin's series (Berrey et al 1990) using non-irradiated allografts, had an overall fracture rate of 16% (43 of 274 cases). Rapid dissolution of the graft (Type I fracture) accounted for 5% (2/43) of the fractures in Berrey's report; a fracture of the shaft (Type II) in 51% (22/43) and a fracture

involving the joint (Type III) in 44% (19/43). Thompson's (1993) series of 35 massive allografts followed for 26 months had a fracture rate of 46% (16/35), of which 88% (14/16) were type II. Allan et al (1991) found a fracture rate of 6% (2/31) in irradiated calcar replacing bulk grafts used in revision hip surgery. Only 1 out of 31 (3%)larger allografts used with a cemented prosthesis sustained a fracture. Some resorption of the graft was noted in 8% (3/35) of massive allografts by Thompson et al 1993 but none led to complete dissolution. Resorption of grafts was noted by Mahoudeau (1970) in 16% (9/54) and by Loty (1990) in 8% (2/25) of lower limb intercalary grafts but 33% (6/18) of humeral reconstructions. Allan et al (1991) found that the use of a cemented prosthesis inside irradiated calcar replacing bulk allograft used in revision total hip replacement reduced the resorption rate from 79% (11/14) to 10% (1/10). Resorption was not seen in larger (non calcar) allografts used with a cemented prosthesis. The authors questioned the use of calcar replacing bulk allograft.

Mahoudeau (1970) reporting Merle D'Aubigne series of non-irradiated grafts had a fracture rate of 4% (2/54). Loty (1990) reported, in a later series from the same institution but with the addition of irradiation, that fracture occurred in 8% (2/25) intercalary lower limb allografts. Donati et al (1993) reported a 15% (17/113) incidence of fracture in a multicentre trial in Europe. Hernigou et al (1986) reported two fractures out of thirty (6.7%) massive irradiated allografts.

The finding that the proximal humerus was a prevalent site for fracture (Gebhart et al (1990) and Mnaymneh and Malinin (1989)) led to the suggestion that fracture may

be related to revascularisation and thus weakening of the graft before adequate remodelling could restore strength to the graft. It was hypothesized that the subchondral bone of the shoulder might predispose to this. However, during re-operations for fracture, the graft is often not revascularised (Thompson et al 1993) and the finding of Berrey et al (1990) that fracture may be associated with non-union suggests that a delay in revascularisation may be a factor in fracture. Plate fixation is associated with a higher incidence of fracture because the graft appears sensitive to the stress riser in the screw holes (Vander Griend 1994, Thompson et al 1993).

From the literature it is hard to establish if irradiated grafts are more vulnerable than non-irradiated grafts to fracture or resorption. It appears that other factors are of more importance to the survival of the graft, such as the method of fixation of the allograft.

Non union of the graft host junction in non-irradiated grafts was reported to be 4.6% by Mahoudeau (1970), 10.3% by Mankin (1983) and 11% by Vander Griend(1994). Non-union rates in irradiated grafts are reported by Hernigou (1986) to be 5 out of 39 (13%) host graft junctions and by Loty (1990) in 5 out of 25 (20%) lower limb intercalary reconstructions. Allan et al (1991) found a non-union rate of 17% in irradiated grafts in revision total hip replacement. The site is important because Loty (1990) also reported non-union in only 5% of 20 proximal femoral reconstructions and 16% of 25 knee reconstructions using irradiated grafts. Vander Griend (1994) found that metaphyseal junctions healed more rapidly than diaphyseal junctions and that non-union was related to a gap of more than 3 millimetres at the junction or some

problem relating to the fixation method.

Impacted cancellous allografts have been used successfully in revision total hip replacement with cemented prosthetic components (Gie 1993, Nelissen 1995). Solid impaction of the allograft cancellous bone is essential to provide structural stability for the new cemented components. Histological examination of retrieved specimens reveals that revascularisation of the cancellous bone is well established by one to two years (Nelissen 1995, Ling RSM 1993). One study has found that cancellous allograft irradiated at 5.0 Mrads (50 Kgray) functions as well as non-irradiated cancellous bone when used in impaction grafting (Bannister 1992).

Conclusions and objectives of the study

The following conclusions can be drawn from this literature:-

1. The most common clinically used dose of irradiation 2.5 Mrads. It will destroy most clinically relevant bacteria. However, there is some doubt if this dosage is sufficient to destroy HIV.
2. Doses of radiation less than 3 Mrads do not significantly weaken the allograft in bending, torsion and compression.
3. Both freezing and irradiation reduce the immunogenicity of allograft when measured by antibody and cell mediated responses. Klinman's work on syngeneic mice suggests that this may not relate to healing of the graft.
4. The literature is divided as to whether or not irradiation has an adverse effect on the incorporation of bone grafts. The work of Urist and Buring suggests that irradiated demineralised bone graft, transplanted into an ectopic site, loses some or most of its osteoinductive capacity. Other work by Weintroub and Reddi contradicts this. It is not clear how these models compare to the real life situation when massive whole bone allografts are implanted.
5. The clinical results of allografts would suggest that other factors such as site of grafting and security of internal fixation may be more important to the success of the graft than the use of irradiation for sterilisation.

From the review of the literature it is clear that clinical infection and transmission of blood borne disease are serious problems following bone transplantation. If there are pathogens contaminating the graft, it seems logical that these should be reduced by

secondary sterilisation. However, it is not clear from the literature what role graft contamination has in subsequent clinical infection. It is probable that the nature of the surgery for which the bone transplantation is done, is more important than minor contamination of the graft, in the development of subsequent infection. It appears to be reasonable practice to decontaminate grafts by sterilisation to reduce bacterial counts to an "acceptable bioburden".

The literature is divided as to the dose of irradiation required to inactivate the HIV virus and so reduce the risk of disease transmission. The commonly used dose of 25 Kgray may not be sufficient Conway et al (1991). More research is required in this field to determine the minimum dose required. At the time this project was undertaken, the most commonly used dose in clinical practice was 2.5 Mrads (25 Kgray), which was thought to inactivate all pathogens and appeared to have little deleterious biomechanical effects.

The large body of experimental work in Urist's laboratory suggested that 2.5 Mrads had deleterious effects on the production of new bone by demineralised bone matrix. The evidence from Weintroub and Reddi (1988) however was not so damming. The clinical data shows little difference between the use of irradiated and non-irradiated grafts, which may be because any subtle effects of irradiation are overshadowed by the problems of graft non-union, fracture and infection. If other problems are more likely to give rise to graft failure, is it of clinical importance if irradiation has a harmful effect? Despite the elegant model of Urist, there has been little attempt in the literature to examine the intact allograft with regard to the effect of irradiation on

healing and performance.

Therefore, the aims of this study were as follows:-

1. To establish the incidence of contamination and infection in a bone bank. Is there an obvious need for sterilisation?

2. To establish an animal model that closely mimics the clinical situation for the study of incorporation of large allografts.

3. Having established the model, to investigate the null hypothesis that irradiation at 2.5 Mrads (25 Kgray) has no deleterious effects on allograft incorporation.

CHAPTER II

STUDY ONE: THE INCIDENCE OF CONTAMINATION AND INFECTION IN THE LEICESTER BONE BANK

Aim

The objective of this study was to determine the incidence of bacterial contamination in bone allografts and the subsequent risk of clinical infection after their re-implantation. To achieve this a review study of the first eighteen months of operation in the Leicester Bone Bank was undertaken.

Introduction

The Leicester Bone Bank is a non profit, hospital based, tissue bank, established in 1989, to provide human allograft material for a variety of orthopaedic surgical procedures. Transplantation procedures are based on the principles employed by the American Association of Tissue Banks (1987) and the European Association of Musculoskeletal Transplantation (1992).

Methods

Donors

Living donors were patients having primary total hip replacements whose femoral heads were retrieved at operation and stored for elective use. All the patients had given fully informed written consent, including consent for HIV blood tests.

Cadaver donors were used for acquisition of long bone and osteochondral grafts.

Consent for cadaver grafts was obtained from the next of kin. Eighteen years was chosen as the lower age limit for grafts to avoid grafts with open epiphyses. The upper age limit for osteochondral grafts was 45 years to ensure that the articular cartilage and menisci were not subject to significant degenerative change. Intercalary and non-osteochondral grafts had an upper age limit of 65 years. Live donor femoral heads had no upper age limit.

Table II.1 (page 53) shows the contra-indications used by this bank. All patients were screened for disease using the investigations listed in table II.2 (page 54). HIV testing of live donors was done preoperatively and, at that time, 3 months later to detect late seroconversion.

Retrieval

The methods of retrieval were in accordance with the guidelines described by Tomford et al (1987). Living donor femoral head grafts were retrieved at total hip replacement under sterile operating theatre conditions. After removal, the femoral head was swabbed for culture and any attached soft tissue removed. The graft was placed in two sterile screw top jars and stored in a freezer at -20 degrees centigrade.

Retrieval of long bones from cadaver always took place in a standard clean operating theatre. Using aseptic techniques the grafts were obtained from the cadaver and passed to a surgeon working at a back table. The grafts were soaked in antibiotic solution (Polybactrin Soluble GU, Wellcome) for 5 minutes and then stripped of soft tissue except ligament, capsule and tendon insertions. The grafts were swabbed for

culture, wrapped in a sterile plastic bag, three layers of sterile towels and another sterile plastic bag, prior to parcelling finally in paper and taping. Osteochondral grafts were treated in the same manner except that the articular cartilage was covered with a gauze soaked in dimethylsulphoxide. All grafts and swabs were clearly labelled.

Storage

In 1989 and 1990 the femoral heads were stored at -20° centigrade but as from 1991 the femoral heads have been stored at -80°C. All cadaver grafts were stored at -80°C. The freezers were alarmed to detect a rise in temperature of 10°C and protected from mains electricity failure or mechanical failure by a carbon dioxide backup system.

Release for implantation

On the basis of the retrieval bacteriological culture results, grafts were labelled as sterile or contaminated. Contaminated femoral heads were discarded whereas contaminated cadaver grafts were irradiated at 2.5 Mrads. All blood results from investigations in table II.2 (page 54) were reviewed and if satisfactory, the graft was passed for implantation and labelled as such. Long bone grafts were radiographed with a ruler prior to implantation to ensure correct size match.

Implantation

At re-implantation the grafts were delivered to the scrub nurse by removing the outer two wrappings for long bone grafts and the outer jar for femoral heads. After

unpacking by the scrub nurse, one implantation culture swab was obtained and the grafts were then washed in a solution of Cefuroxime (Glaxo) while thawing. The grafts were shaped, implanted and fixed depending on the operative procedure involved.

Review

The documentation for each graft was kept in a separate file and in addition donor and recipient details were stored on a personal computer database. All grafts retrieved in 1989 and 1990 were subjected to review in October 1991 to evaluate the first eighteen months of operation. The case notes of all patients who received a graft were reviewed and in those cases where the grafts were implanted at another centre away from Leicester, the surgeon in charge of the case was asked to fill in a questionnaire after reviewing case notes.

Clinical sepsis was divided into wound infection and deep sepsis. Wound infection was defined as erythema, discharge and a positive culture swab from the wound. Deep sepsis was defined as sepsis confirmed on a culture swab, which on surgical drainage, was found to extend down to the graft. This required removal of the graft.

Results

Live Donor Femoral Head Grafts

One hundred and sixty one femoral heads were retrieved between 1st July 1989 and 31st Dec 1990 inclusive. Subsequently one hundred and three femoral heads were re-implanted in 59 patients. The remaining 58 heads were not used for the reasons shown in Table II.3 (page 54).

The mean age of the recipients was 60 years (range 21 - 80) and the indications for grafting are shown in Table II.4 (page 55). Six femoral heads (6%) were not swabbed for culture at the time of implantation. Culture of the femoral head at time of re-implantation was positive in 5 out of 97 cases swabbed (5%); one case with Acineto lwoffi and the other 4 with Staphylococcus albus.

Two of the re-implantation cases contaminated with Staphylococcus albus were followed by clinical sepsis after re-implantation. One case, a man aged 64 with rheumatoid arthritis had removal of a total knee replacement 6 years previously because of infection with Staphylococcus aureus. His arthrodesis did not unite requiring internal fixation with plates and bone grafting with 4 femoral head allografts (one of which was contaminated with Staphylococcus albus). This was followed by a deep sepsis with Serratio liquefaciens and Acineto calcoaceticus which required surgical removal of the graft and plates. The second case was a revision total hip replacement which resulted in a wound infection with mixed bacterial flora.

Two patients (3%) had deep sepsis, one of whom has been discussed already. The other was in a lady aged 83, who had a subtrochanteric femoral fracture treated with screw plate fixation and a femoral head allograft. She developed early deep sepsis with coliforms and faecal streptococci, requiring removal of the graft. Five patients (8%) had wound infections; three with a mixed flora and two with Staphylococcus aureus. One required surgical drainage and all settled after appropriate antibiotic therapy.

The median storage time in the bone bank from retrieval to implantation was 7 months. Femoral heads were discarded after 12 months storage at -20°C because they were considered to have exceeded their shelf life.

Cadaveric Massive Grafts

During the study period there were six cadaveric donors who provided 22 grafts. Four of these 22 grafts (18%) were contaminated at the time of retrieval, three with Staphylococcus albus and one with Staphylococcus albus and diptheroids. These contaminated grafts were all irradiated. Nine of the grafts have so far been implanted in 9 patients; 4 as alloprostheses, 3 as intercalary and 2 as osteochondral grafts. Three grafts have been used in revision hip replacements and 6 in tumour resections.

There was one clinical infection in the study period, in a lady who had an alloprosthesis for a chondrosarcoma of the proximal femur. She developed deep sepsis with Escherichia coli and faecal streptococci which necessitated disarticulation at the hip. There were no other infections.

Discussion

This audit study has shown that there is a high contamination rate of grafts, from both live and cadaveric donors. This has been reported by other authors (Lord et al 1988, Veen 1994) and is one of the disadvantages of the fresh frozen method of preservation of grafts. The high rate of contamination in femoral head grafts was surprising in view of the clean nature of hip replacement surgery. No obvious factor was found to explain this. This study only used one culture swab to determine if contamination was present which may underestimate the problem (Gristina and Costerton 1985 and Veen 1994). Veen (1994) recommends culture of the bone itself with extended subcultures. The use of such contaminated grafts would be contraindicated without sterilisation by irradiation, heat or ethylene oxide.

This study found that a small number of grafts that had a negative culture at retrieval were found to grow bacteria on the swabs taken at the time of re-implantation. The use of only one culture swab may lead to spurious results if contaminants are introduced at surgery or in the microbiology laboratory. Although the result of this re-implantation swab will not be available for a minimum of 24 hours after surgery it may help with antibiotic choice, both for prophylaxis and treatment. However, transplantation of a contaminated graft is unlikely to be sterilised by systemic antibiotic therapy in view of the avascularity of the graft. In those cases with clinical sepsis, the re-implantation culture result may help determine if the graft was responsible for the clinical infection.

The clinical sepsis rate for femoral head allografts used in this study was 11%, unlike the negligible rate found in a retrospective questionnaire study by Tomford et al (1990). One factor that may have influenced the sepsis rate in this study is that many of the grafts were used in re-revision arthroplasty operations which have been reported to have an infection rate of 8% without the use of allograft (Kavanagh 1991). The infection rate in large allografts used in this study was also 11%, which is similar to that reported by Lord et al (1988).

This study highlighted the large wastage of femoral heads which it would be beneficial to minimise. One third of the femoral heads had to be discarded. Twenty-two were thawed and not implanted by surgeons. This wastage can be avoided by not defrosting a femoral head until the surgeon is absolutely sure he needs the graft. Grafts can be kept frozen on the day of operation at minus 20°C in a freezer in theatre and then thawed quickly in warm saline when required. If not used, then the grafts can be returned to the bone bank for further storage at minus 80°C. Fourteen (24%) femoral heads were discarded because their donors did not have their second HIV blood test even though a blood request form was attached to the notes of all bone bank donors with instructions for the blood test to be done at their three month outpatient appointment. This wastage can be avoided by employment of a domiciliary phlebotomist or by asking the patient and general practitioner to arrange for the blood tests to be performed.

Six (10%) femoral heads were found to have come from patients having a conversion of a hip fracture fixation to total hip replacement. These patients were considered by

the medical director of the bone bank to carry a high risk of bacteriological contamination and were therefore discarded. Previous hip surgery is now considered a contra-indication to donation. Six (10%) of the femoral heads exceeded the shelf life of six months at minus 20°C. A change to femoral head storage at minus 80°C, with acceptable storage times of up to 5 years (American Association of Tissue Banks 1987), means it is unlikely that any further femoral heads will be wasted because the shelf life is exceeded. Furthermore, many banks now freeze to minus 80°C to enable a six month quarantine so that the donors can be retested for HIV.

It is important that bone banks audit their performance carefully. The prospective collection of data relating to each donor, the collection and storage of allografts and recipients is essential. Evidence from one tissue bank shows how important this is for tracing recipients in the rare event of a donor having HIV infection although the individual was seronegative at retrieval (Simonds et al 1992). At the time of the study, the Leicester Bone Bank relied on a traditional filing cabinet approach to data collection and storage. They have since changed to storing data on a microcomputer which should make the task of audit much easier in future. The use of a database may make the achievement of second HIV blood tests more reliable.

Clinical infection and performance of the implanted grafts are more difficult to audit. In this study, this was achieved retrospectively from case notes, which is far from ideal. Collecting this data prospectively would demand that each surgeon who uses transplanted bone has to fill in yet more forms to be returned to the bone bank and compliance with this is likely to be uncertain.

The success of the Bank in Leicester has hinged on the enthusiasm of the personnel running it. Tasks such as identification of donors, packaging of grafts by theatre staff, blood and microbiological investigations and collection of these results are all extra to the demands of an ordinary orthopaedic unit. Any sizeable bank will require at least one full time secretary/ assistant for collection and storage of the data alone. The running of the bank requires medical supervision, particularly with regard to exclusion of some inappropriate donors and decisions about sterilisation of contaminated grafts (Tomford et al 1989). Lastly, the retrieval of large cadaver grafts depends on a qualified team of surgeons and assistants as in other organ retrieval programmes. The sterile retrieval of grafts and reconstruction of the cadaver are skills that must be mastered before embarking on retrieval.

This pilot study emphasises why the difficult task of bone bank audit is important. By standardising how the data is collected in different banks, comparative audit studies will enable further refinement of working practices.

The rate of contamination was higher than expected. This study suggests that there is a need to look at methods of sterilisation of bone grafts so that grafts which are contaminated at procurement may be used for transplantation.

Tables

TABLE II.1

Contraindications for bone donation

Age less than 18

Chronic drug abuse

Chronic or high dose steroids

Exposure to toxic chemicals

Long standing insulin dependant diabetes

Malignancy

Treatment with growth hormone

Unexplained jaundice or hepatitis

Dementia

Chronic neurological disease

Recent infection

Recent immunisation with live vaccine

At risk factors for HIV infection

TABLE II.2

Screening investigations of potential donors

Live Donors	Cadaveric Donors
Hepatitis B and C	Hepatitis B and C
VDRL	VDRL
HIV antibody	HIV antibody
3 Month HIV antibody*	Blood cultures
	Cytomegalovirus

* now done at six months

TABLE II.3

Reasons for discarding the 58 unused femoral heads (number and percent).

REASON	**No.**	**%**
Thawed and not implanted	22	38
Failure to get 2nd HIV blood test	14	24
Prolonged shelf life	6	10
Previous implant hip surgery	6	10
Post operative death before 3 month HIV test	3	5
Contaminated at retrieval	2	3
Other	5	9

TABLE II.4

Indications for re-implantation of 103 femoral heads in 59 recipient patients (number and percent of patients).

INDICATION	No	%
Joint reconstruction		
Revision total hip replacement	35	59
Revision total knee replacement	10	17
Fracture around a total hip replacement	4	7
Primary total hip replacement	1	2
Total	50	85
Fractures		
Primary fixation	2	3
Non-union	4	7
Total	6	10
Arthrodesis	2	3
Tumour	1	2

CHAPTER III

PRELIMINARY EXPERIMENTS TO ESTABLISH THE ANIMAL MODEL

Aim

This chapter reviews the initial animal experiments which were undertaken to establish a model which could be used to measure new bone formation around large bone allografts.

Introduction

The animal chosen for the work was the New Zealand White rabbit for several reasons. There are many reports of the use of this animal for allograft and fracture studies. The animal is inexpensive and the laboratory was familiar with preparation of tibial specimens for light microscopy.

The previous work by Urist and his coworkers had concentrated on demineralised bone matrix transplanted into the abdominal wall of rats and rabbits. This situation is removed from what happens in clinical practice and it was the aim of this project to study the healing and incorporation of a bone graft in a model that closely resembles the clinical situation. For this reason it was decided to examine the healing of an intercalary allograft in the tibia. The aim was to model the situation in which a bone tumour would be excised with its periosteum and replaced by a structural bone graft. A routine method of internal fixation of the graft would involve use of an interlocking intramedullary nail which would achieve axial and rotatory stability. In place of locking screws, a modification of the intramedullary wiring technique of

Phillips and Hooper (1985) was developed to achieve stability and compression at the osteotomy sites. Excision of the periosteum was considered necessary to avoid its influence in the repair process in the region of the intercalary graft segment.

The ability of bone grafts to incorporate depends on two important processes. Grafts can produce bone from the surrounding tissues by bone induction and act as a scaffold for bone conduction (Burchardt 1983). Bone induction occurs early on in graft incorporation whilst conduction is a gradual process occurring later. One of the aims of this experiment was to quantify bone induction and conduction in vivo and so compare irradiated allograft incorporation with that of non-irradiated allograft using an autograft control.

Standard methods of quantification of bone depend on histomorphometry such as bone volume, osteoid volume, osteoid surface. These measurements provide a static estimate of the quantity of bone at a given time when the measurements are taken. They do not, however, give any information on the rate of new bone formation which can be measured in vivo using tetracycline labelling.

Tetracycline binds to bone during the process of mineralisation (Milch 1958). Ultraviolet light at a specific wavelength range, used to illuminate the labelled specimen, causes emission of light of a different wavelength which can be observed by careful utilisation of optical filters. Studies comparing standard staining for mineral (e.g. Von Kossa stain) and tetracycline deposition show that tetracycline is reliably taken up by mineralising tissues (Villaneuva 1983). Its advantages are its specificity

for this process and its fluorescence under ultraviolet light. Giving two doses of tetracycline at different times allows measurement of rate of mineralisation (Frost 1976). Provided there is no defect in the mineralisation process (eg vitamin D deficiency) then this rate of mineralisation matches the rate of bone apposition.

Most tetracyclines fluoresce under ultraviolet light. Demethylchlortetracycline was chosen for this experiment because it is reported to have a bright florescence (Canalis 1982), is readily available and can be made up to form an aqueous solution for intramuscular or subcutaneous injection (important for lower animal work). The magnitude and timing of the doses has been investigated by other authors. Extremely high doses of tetracycline should be avoided as they have an inhibitory effect on mineralisation (Harris 1968). The interval between the labels should be less than 3 weeks as a longer interval will result in few trabeculae bearing two labels. The interval between the last label and biopsy should be longer than 24 hours to avoid washout of the label during processing particularly if aqueous fixatives are used (Revell 1985). Canalis (1982) suggests that a suitable dose for demethyl-chlortetracycline in experimental work is 75 mg/kg, given as an intramuscular or subcutaneous injection. The use of parenteral administration of the tetracycline means that only one dose is required unlike orally administered tetracycline in human and higher animal work, when two to three days administration is the norm.

Ultraviolet light at a specific wavelength range, used to illuminate the labelled specimen, causes emission of light of a different wavelength, which is kept to a specific wavelength range by the use of a barrier filter. Traditionally, the illuminating

ultraviolet light was passed through the specimen (transmitted light) which had to be processed undecalcified to avoid washing out of the fluorescent label with the mineral. Fixation of the specimen was done in methacrylate following which sections were cut roughly (20 to 40 microns thick) and then ground to a thickness that light could pass through (8-10 microns). The soft tissue components process well but commonly there was cracking of the mineralised bone and these techniques were found to work best on small specimens. In addition, the grinding process was laborious and time consuming.

Canalis (1982) described a method of preparation of specimens which relied on the ultraviolet light being incident on the section rather than transmitted through the section. This meant that the sections did not have to be so thin as that required for transillumination. However, a technique for plastic processing of human bone with a polyester resin (Polymaster 1209 AC supplied by Bondaglass Voss Ltd, UK) has been described by Mawhinney and Ellis (1983) which overcomes the problems of sections disintegrating when cut thin enough for transmitted light. This technique was used by Pallet et al (1986) for cemented hip joint replacement specimens and by Mitcheson et al (1988) for human iliac crest biopsies with tetracycline labelling. This processing technique had not been evaluated for fluorescent microscopy in animal bone.

First cadaver study

A simple study was performed to perfect the osteotomy and assess the best method of fixation of the allograft.

Method

The initial study was performed on five cadaver rabbit tibias which were stripped of soft tissue and mounted in a vice. Transverse osteotomies were made using a reciprocating saw, 1 cm proximal and 1 cm distal to the inferior tibio-fibular synostosis, thus excising a 2 cm segment. An entry hole was made into the upper end of the proximal tibia using a 2.0mm drill, 0.5 cm medial and proximal to the tibial tubercle. A 1.6mm Kirschner wire was introduced through the proximal tibia, the graft and the distal segment. The fixation proved to be rotationally unstable despite various attempts to improve the fixation using thicker wires and introducing a second wire into the proximal segment where the tibia is widest (Oni 1987).

Since satisfactory fixation could not be achieved, it was decided to use an intraosseous wiring technique (Phillips and Hooper 1985). Before insertion of the longitudinal K-wire as described above, drill holes were made in the ends of the graft and the adjacent tibia to accept a wire suture. A 15 cm length of 1.6 mm stainless steel wire suture was passed through the superior drill hole of the graft and the corresponding drill hole of the proximal segment of the recipient tibia so that the free ends were medial. A similar piece of wire was passed through the distal hole in the graft and the hole in the distal recipient tibia. The longitudinal Kirschner wire was tapped gently home ensuring that it passed down the tibia, through the graft and into

the distal tibia. The ends of the wire sutures were then twisted under tension to close the recipient tibia/ graft junctions. Wires thicker than 1.6mm caused the bone to break as the wire was tightened; hence 1.6mm wires were used. (See Figures III.1, page 65 and III.3, page 66, which show the reconstruction procedure in live animals.)

The Kirschner wire was tapped fully home and the site of entry into the tibia was marked. The wire was then backed out a little to allow the wire to be cut at this point and then tapped home again. This ensured that the wire was left protruding by 2 to 3mm from the entry hole contributing to the stability of the reconstruction.

Results

The longitudinal K-wire achieved longitudinal fixation. The osteotomy sites were compressed and made rotationally stable by tightening the two wire sutures. The final reconstruction was stable in each case.

Preliminary live animal study

This study was designed to assess the healing of the osteotomy in vivo and to assess the use of fluorescent labels to label new bone formation.

Methods

Nineteen adult female New Zealand White Rabbits of weight between 3.0 and 5.5Kg were used.

Anaesthesia:

The animals were sedated with an intramuscular (im) injection of 10mg of midazolam. Induction of anaesthesia was achieved with an intravenous injection of Hypnorm, 0.1 ml/kg (Fentazyl 0.315 mg/ml and fluanisone 10 mg/ml) through an ear vein. The anaesthesia was maintained with inhalational halothane/oxygen mixture at an average of 4% halothane applied through a face mask. At the end of the procedure the animals were given an intramuscular dose of 0.3 mg buprenorphine for analgesia which was repeated for post operative analgesia.

Preparation of the limb

The fur was shaved from the right hind limb, from the ankle joint to the hip joint. The foot and the lower trunk were wrapped in plastic sheeting to exclude fur from the operative field. The skin was prepared with betadine and chlorhexidine skin preparation solutions. Sterile towels were then used to drape the operative field and exclude unsterile areas.

Incision and exposure

A longitudinal incision was made on the anteromedial aspect of the tibia, from just below knee joint to 1 cm proximal to the ankle joint. The incision was deepened through the fascia to expose the subcutaneous border of the tibia.

Procedure

The muscles were dissected off the lateral and medial sides of the tibia by sharp dissection with a scalpel, staying outside the periosteum. MacDonald's dissectors were placed behind the tibia to protect the soft tissues. A scalpel was then used to make two transverse marks on the surface of the tibia, 2cm apart, in the midportion of the tibia. The lower mark was 5mm below the inferior junction of the tibia and fibula. Drill holes were then made with an electric dental drill, fitted with a 2mm drillbit, 5mm on either side of the marks on the tibia, for later placement of wire sutures. The drill holes were made through both cortices, from the medial surface side. A reciprocating bone saw (with saline irrigation) was then used to make the two transverse osteotomies at the sites previously marked. Following this, the bone segment was still attached by the fibula and this was broken at its junction with the tibia. Any remaining soft tissue attachments posteriorly were removed by sharp extraperiosteal dissection.

The segment of bone was then removed, stripped of its attached periosteum and the marrow removed from the core. It was either set aside for immediate re-implantation if the animal was an autograft control or stored immediately in a sterile container for freezing to minus 80 degrees celsius.

Reconstruction

A further drill hole was made on the antero-medial aspect of the tibia opposite the insertion of the patellar tendon. this hole was enlarged with a scalpel to allow the passage of a 1.6 mm Kirschner wire into the medullary cavity of the proximal tibia. Attention was then turned to the graft which was laid in the defect in the recipient tibia, in the correct orientation. The reconstruction then proceeded as described above in the cadaver study. The ends of the 1.6mm wire sutures were buried in soft tissue (Figure III.1). The stability of the fixation was checked prior to closure by applying manual varus/valgus and rotational forces.

Closure

The fascia and skin were each closed with a continuous 2/0 polyamide "Vicryl" suture. The wound was sprayed with an adhesive plastic spray (Opsite). The first two animals operated on initially did not receive any external splintage but it was observed that they appeared to have inadequate fixation to bear the body weight and the legs angulated. For this reason all subsequent animals were splinted in a padded lightweight cast (Scotchcast Plus, 3M) (Figure III.2). This was applied from the upper thigh to the toes, with the knee and ankle joints flexed to avoid the cast slipping off. Plenty of padding was included at the knee and ankle to avoid undue pressure at these points. Although this casting material was harder to mould and more expensive than plaster of paris, it was found to be the best material to resist the attempts of the animal to chew the cast. All animals were able to weight bear in the cast although the majority held the leg externally rotated at the hip with the toes pointing to the side.

Figure III.1. Intra-operative photograph of a rabbit tibia after completion of the reconstruction using an allograft. The graft has been secured by a longitudinal intramedullary wire (the end of which can be seen protruding proximally, indicated by the arrow) and two intraosseous wire sutures.

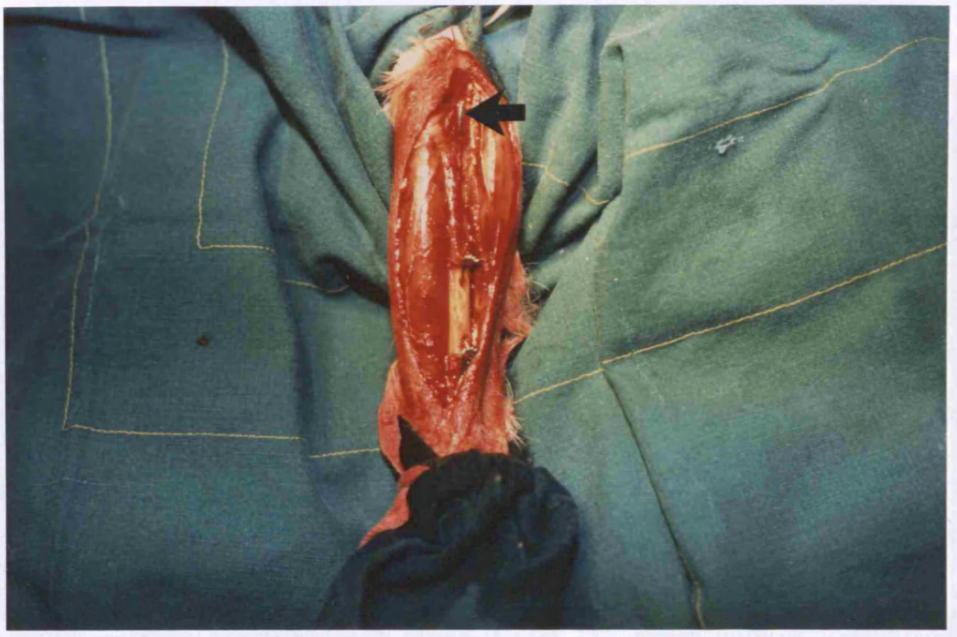


Figure III.2. Photograph of the rabbit tibia after completion of the operation and immobilisation in a lightweight cast.

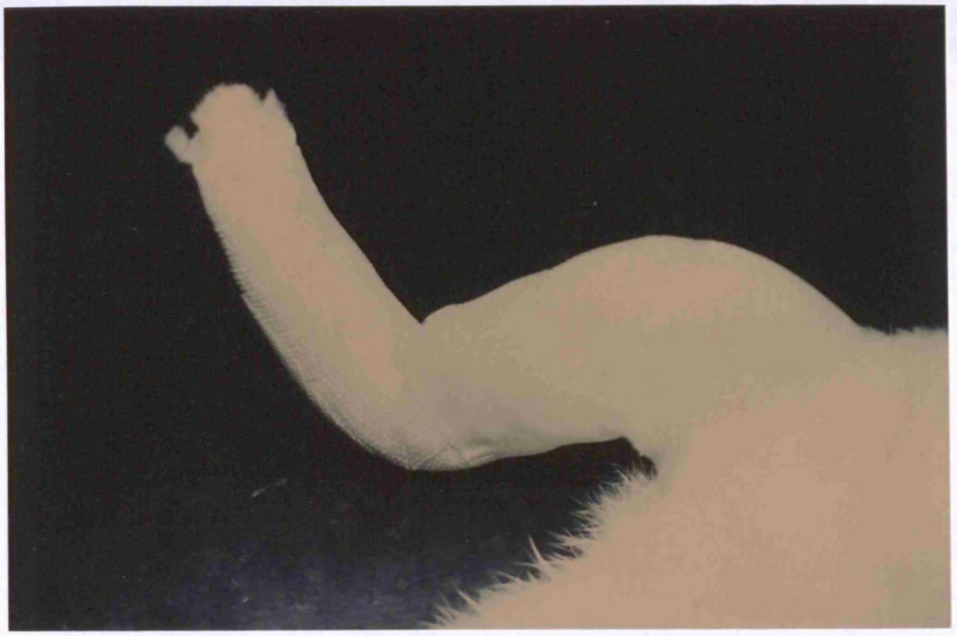
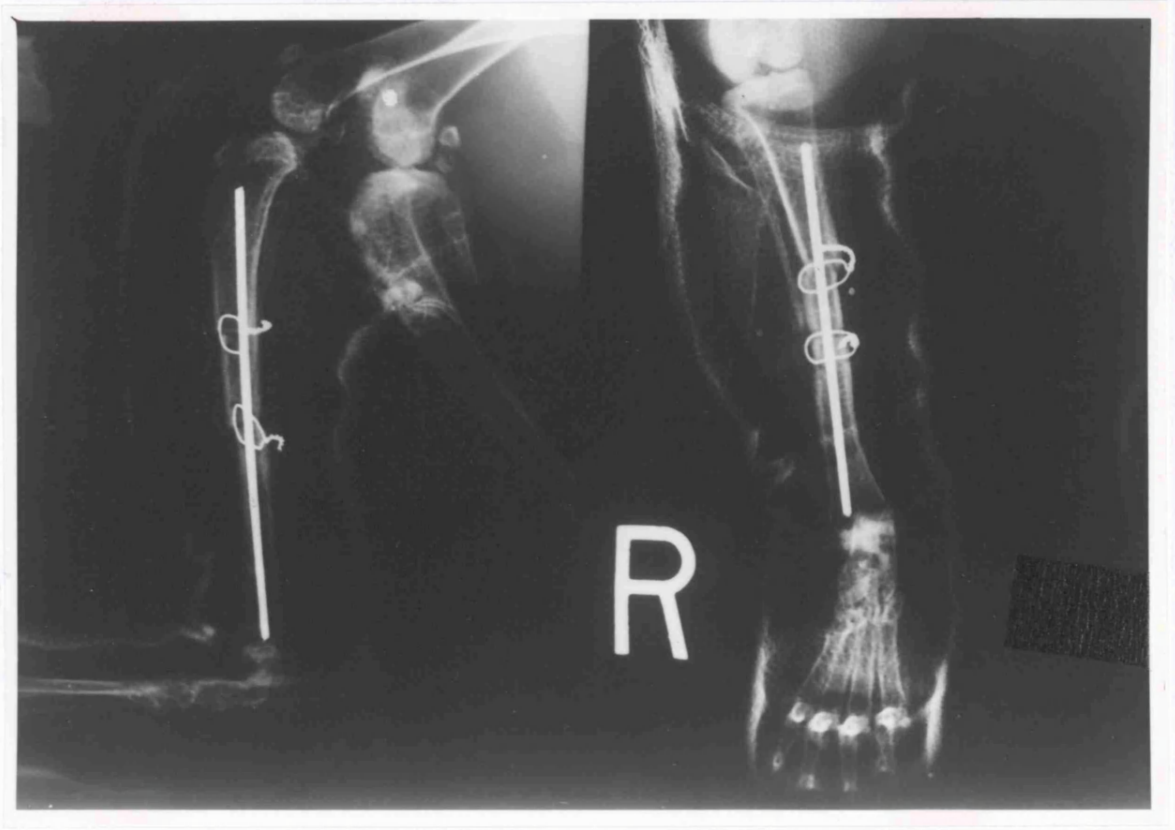


Figure III.3. Photographs of AP and lateral radiographs of a rabbit tibia performed two weeks after surgery. The intramedullary wire and intraosseous sutures can be seen. Compare with figure III.1.



Post-Operative care

The animals were given repeated intramuscular injections of 0.3 mg buprenorphine (Temgesic) B.D., over the first 5 days, for analgesia. The animals were kept caged separately, fed a standard diet, and a daily clinical check was carried out. At two weekly intervals radiographs (Figure III.3) were taken. During the course of the experiment, it was often necessary to reinforce or replace the cast when the animals had chewed their casts. Three weeks prior to culling the animal it received a subcutaneous injection of a demethylchlortetracycline (Canalis 1982) at a dose of 40 to 75 mg/kg to determine the most effective regime. One week prior to cull it received a second injection of demethylchlortetracycline at a dose of 40 to 75 mg/kg. Four animals received a subcutaneous injection of xylenol orange fluorochrome (40mg/kg) instead of the second dose of demethylchlortetracycline to assess if this agent could be used to provide fluorochromes of different colour.

Graft retrieval

The animals were culled between four and twelve weeks (tables III.1 and III.2, pages 74 & 75) by an intravenous injection of an overdose of phenobarbitone. The cast was removed from the leg and the wound reopened to expose the tibia. The entire tibia was removed and stripped of excess soft tissue by sharp dissection.

Preparation of the specimen

The stability of the proximal and distal tibia/graft junctions were assessed manually by applying a torsional force. The wire sutures and the Kirschner wire were removed prior to high resolution radiographs being made on a Faxitron (Hewlett-Packard) at settings of 27 Mv and 2.5 Ma using CEA Singul XRP X-Ray film. Evidence of bone union was assessed on this radiograph and compared to those taken weekly before the animal was killed.

The specimen was then cut, using a water lubricated band saw, so that two transverse discs of bone, each approximately 3 mm in depth, were removed from the centre of the graft. The excess recipient tibia was trimmed off each end to leave two segments of tibia, approximately 1.5 cm in length, each containing a tibia/ graft junction. Each of the segments was then split in a longitudinal fashion down the medullary canal of the tibia and graft. This provided two sets of three specimens; a transverse section and a longitudinal section of the proximal and distal junctions. One set of specimens was then processed for haematoxylin and eosin staining and the other for fluorescent microscopy (See Appendix 1 and 2).

Results of preliminary live animal studies

The operative technique was mastered and good fixation was obtained. Regular radiographs in cast showed that the legs maintained good alignment. All grafts were assessed clinically by rotation after removal of soft tissues for evidence of bony union

and the results are shown in Table III.1 and III.2 (pages 74 & 75). Clinically all allografts and all autografts had united at the proximal end by four weeks. Union distally was more variable but appeared to be related to how long the graft had been implanted.

The radiographs revealed that the fibula united to the graft in all cases and thus stabilised the proximal osteotomy site (Figure III.4). This meant that detection of union proximally by clinical means was inaccurate because the proximal site might not move on stressing due to union of the fibula to the graft, without actual union of the proximal osteotomy site. On some occasions a bar of bone connected the fibula to the distal fragment bypassing the graft.

Analysis of the AP and lateral fine detail faxitron radiographs of all specimens was therefore undertaken. A three point radiographic scoring scale was used: union, definite nonunion and uncertain at both the proximal and distal host/graft osteotomy sites (Tables III.3 and III.4, pages 76 & 77). The presence and site of fibular union in relation the graft was also assessed to see how this affected proximal union. In all but two cases the fibula united to the proximal or middle portion of the graft. In the remaining two cases the fibula appeared to unite to the distal osteotomy or distal fragment, bypassing the graft.

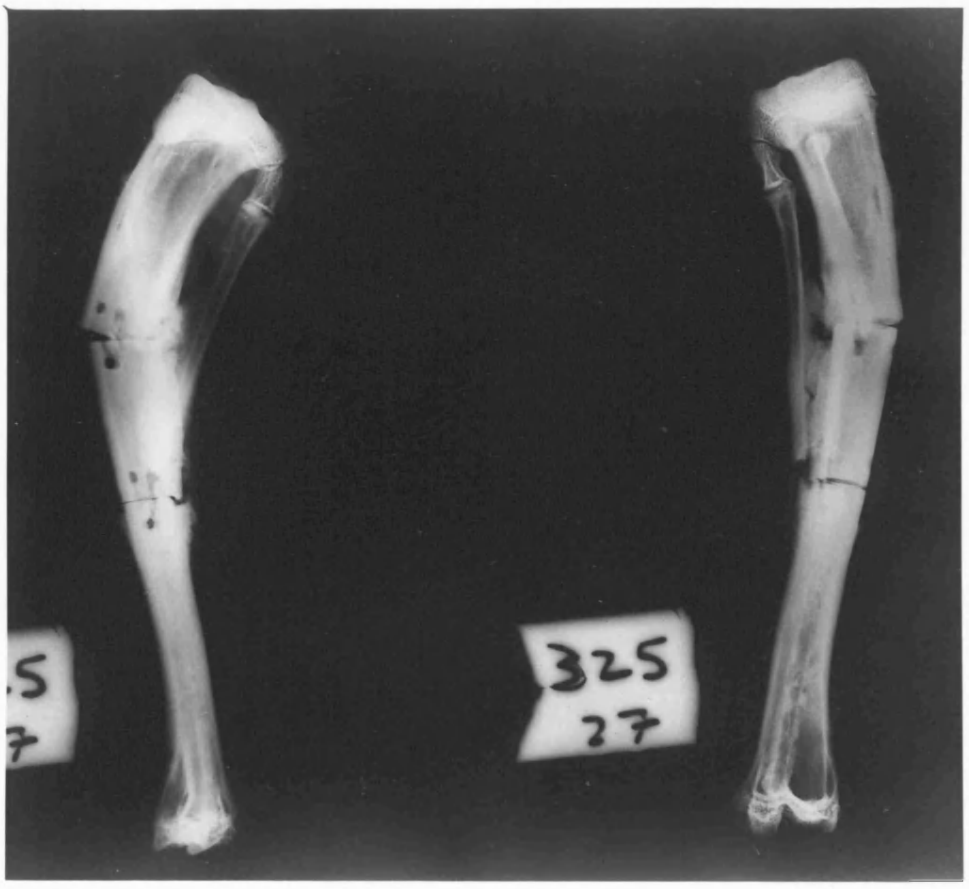
The radiograph results suggested that the fibula was confounding the results, in that union of the fibula to the graft stabilised the proximal osteotomy and encouraged union. The proximal osteotomy site was therefore united in 90% of cases by 6 weeks

and there was little difference between autograft and allograft. At the distal osteotomy site all of the autografts were united by six weeks but only one allograft was definitely united and that was at twelve weeks.

There appeared to be reasonable agreement between clinical and radiological evidence of union, except in cases of proximal host/graft non-union where the fibula was united to the graft. Clinical testing was found to be more finite in the sense that there was either evidence of rotation at the osteotomy site or not. Radiographs were difficult to interpret because callus formed by the periosteal reaction around the host bone cupped the end of the graft. It was often difficult to decide if the callus had united to the graft and 7 out of 38 (18%) osteotomy sites had to be categorised as uncertain.

Figure III.4.

Photograph of AP and lateral Faxitron radiographs of a rabbit tibia specimen with graft after removal of fixation wires. The fibula can be seen uniting to the graft and therefore stabilising the proximal osteotomy site despite there being modest evidence of union at that site. This gave the clinical impression of union at the proximal site.



Fluorescent microscopy

Clearly defined labelled new bone was only visible after tetracycline labelling. The xylenol orange did not appear to label the bone or was not visible using the filter systems tried with the microscope and this was therefore abandoned in later animals. The tetracycline label was clearly seen in both in the callus around the osteotomy and also in the remodelling of the tibial cortex. Those animals given two injections of demethylchlortetracycline had clearly identifiable double labels suitable for estimation of the rate of new bone formation. The optimal regimen for labelling appeared to be a subcutaneous injection of demethylchlortetracycline 60mg/kg three weeks prior to cull followed by a second dose of 75 mg/kg one week prior to cull. By eight weeks abundant new bone was observed around the grafts at the osteotomy sites. Only the autografts showed evidence of revascularisation of the graft with new bone formation by osteoconduction.

Further cadaver study

The influence of the fibula was unexpected. As it was confounding the results, it was decided to make the resection distal to the fibula and so deal with only one bone, i.e. have a true intercalary graft. Two cadaver rabbit tibias were therefore used. A 2 cm section of tibia was marked out, with the proximal limit being just distal to the tibio-fibular synostosis. The drill holes were made as before and the graft was removed using a reciprocating saw. The reconstruction was made as before and at the end of the procedure the grafts were found to be stable. Although the reconstruction was close to the ankle joint there was sufficient room to achieve stable fixation. Good fixation was helped by two factors:- (1) the tibial canal is relatively cylindrical giving

a snug fit around the longitudinal Kirschner wire and (2) driving the K-wire into the distal end of the tibia and leaving the proximal end just proud of the entry hole gave added fixation at the ends of the wire.

Discussion

The preliminary experiments established the model. A two centimetre defect could be created in the rabbit tibia and replaced by an autograft or allograft which was internally fixed using the intramedullary wiring technique. External support for the leg was required. The defect had to made below the fibular synostosis to avoid influence of the fibula in the healing process.

The results suggested that by eight weeks the healing process would be well under way but incomplete. The majority of the proximal osteotomies and the autograft distal osteotomies would be healed. The allograft distal osteotomies might not be healed by this time. The processes of osteoinduction and conduction could be observed and measured. Future study of the healing of grafts could therefore be over a eight week period but with the knowledge that this provided only a snapshot of a continuous process.

Demethylchlortetracycline as a fluorescent label performed well (Canalis 1982). The processing of the specimens worked well and preparation for fluorescence microscopy was achieved without any major problems (Mawhinney and Ellis 1983). This technique appeared to be suitable to the preparation of animal diaphyseal specimens.

Table III.1

Clinical results of bone union at proximal and distal osteotomy in those animals that received **autograft**. S = solid union, F = fibrous union.

Time to cull (weeks)	Number of animals	Proximal osteotomy	Distal osteotomy
4	3	SSS	FFF
5	2	SS	FF
6	1	S	S
7	1	S	F
8	1	S	S
11	1	S	F
12	2	SS	SS

Table III.2

Clinical results of bone union at proximal and distal osteotomy in those animals that received **allograft**. S = solid union, F = fibrous union.

Time to cull (weeks)	Number of animals	Proximal osteotomy	Distal osteotomy
4	3	SSS	SFF
5.5	1	S	S
6	2	SS	SS
11	1	S	F
12	1	S	S

Table III.3

Analysis of faxitron radiographs taken after cull of the animals that received **autograft**. S = solid union, F = fibrous union, ? = uncertain. The two cases labelled with * had union of the fibula to the distal fragment with non-union of the graft at the distal osteotomy site.

Time to cull (weeks)	number of animals	proximal osteotomy	distal osteotomy
4	3	SSS	FF?
5	2	F?	F?
6	1	S	S
7	1	S	S*
8	1	F	S
11	1	S	S*
12	2	SS	SS

Table III.4

Analysis of faxitron radiographs taken after cull of the animals that received **allograft**. S = solid union, F = fibrous union, ? = uncertain.

Time to cull (weeks)	number of animals	proximal osteotomy	distal osteotomy
4	3	FSF	?FF
5.5	1	S	?
6	2	SS	??
11	1	S	F
12	1	S	S

CHAPTER IV

A STUDY OF THE EFFECT OF IRRADIATION ON BONE ALLOGRAFT INCORPORATION.

Aim

Having established the animal model, the purpose of this experiment was to use it to study the effect of irradiation of the allograft on incorporation. Tetracycline double labelling would allow estimation of the rate and the amount of new bone formation, enabling quantification of any effect of irradiation on graft healing.

Introduction

Double labelling with tetracycline of bone permits measurement of the distance between the labels using a calibrated eyepiece to determine bone appositional rate. This measurement is performed at repeated points along each double labelled surface (Frost 1976; Frost and Meunier 1976; Titlebaum and Nichols 1976). The measured distance between the two labels will vary according to the angle of section of the trabeculae. There is a correction factor to allow for the obliquity of the section through the trabeculae (Frost 1976).

It is necessary to make a sufficient number of measurements to reduce the standard error of measurement. One way of achieving this is to calculate the mean value after a given number of measurements, count more fields and recalculate the mean value.

Eventually the mean value settles to a constant level described as the nominal value (Revell 1985). Since there was no previous data to compare with, this would be the method used in this experiment.

There are many parameters that can be measured to gain an estimate of the amount of bone that has been laid down but the most relevant may be measurement of the surface area of new bone. In 1847, the geologist Delesse put forward the principle that in a rock composed of many minerals, the area occupied by any given mineral on the surface of a section of the rock is proportional to the volume of the mineral in the rock (Aherne 1982). The technique of point counting allows easy measurement of the fraction of a given area of a substance made up of one of its components. By the principle of Delesse, this fraction of the surface area allows estimation of the fraction of the volume occupied.

Materials and methods

Twelve mature New Zealand White rabbits weighing between 3.47 and 3.84 kg were operated upon in the manner described in Chapter III (page 62). A two centimetre defect in the lower tibia was created, the upper osteotomy performed below the synostosis of the tibia and fibula (page 72). The reconstruction of the defect was performed as previously described and the leg was immobilised in "Scotchcast" post operatively.

Study Groups

There were three study groups of four animals. Group one had the two segment of tibia reconstructed using their own two centimetre segment of tibia replaced as an autograft. The second group had the defect reconstructed using allograft which had been irradiated at 2.5 Mrads and stored, frozen at -80°C. Irradiation of the graft was undertaken as part of a larger load of human bone allograft being irradiated by the Leicester Bone Bank at a controlled dosage of irradiation in an industrial plant (Isotron, Swindon, UK). Group three had a reconstruction using an allograft stored frozen at -80°C, but not irradiated. Radiographs of the operated leg were performed at two, four and six weeks.

All animals were given a subcutaneous injection of demethylchlortetracycline 60mg/kg three weeks prior to cull followed by a second dose of 75 mg/kg one week prior to cull. All animals were culled at eight weeks. After killing the animals, the tibiae were removed and the stability of the proximal and distal osteotomy sites was assessed manually. Faxitron radiographs were taken after removal of the fixation wires. Specimens were then prepared for H&E staining and fluorescent microscopy (Appendices 1 and 2).

Histology

Each animal provided a proximal and distal longitudinal and a transverse section for H&E and fluorescent microscopy. One good section from each block was examined using an Olympus BH2 microscope (Olympus, Japan). The longitudinal sections were taken from the middle of the host and graft. The transverse sections were through the

middle of the graft. The H&E sections were examined at x40 and x100 magnification. For fluorescent sections, the fluorescent microscopy attachment was used including an excitation filter (20UG1) with bright lines at 334 and 365 nm, a dichroic mirror (DM400) and a barrier filter (17L420) permitting wavelengths longer than 420 nm. Although Canalis (1982) reported that the ideal excitation wavelength should be of the order of 420-460 nm which causes a peak fluorescent wavelength of 480-660 nm, the regime used above was found by trial and error, with a range of Olympus filters, to be suitable for the specimens studied. Magnification of 250 and oil immersion were used for the measurement of rate of bone formation. Magnification of 125 was used for measurement of fractionated amount of new bone formation.

Method of measurement of rate of new bone formation

Each section was examined in turn for the presence of double, sequential labels (Figure IV.1). Clearly defined double labels, seen end-on like a target, were measured to avoid confusion with two adjacent areas of bone labelled at the same time. The distance between the start of the first label to the start of the second label was measured for each target double label, using a linear scale eyepiece graticule. However, trabeculae are not perfectly straight cylinders of bone and the plane of section often crosses trabeculae obliquely, giving rise to oval shaped targets. Therefore, it was decided to measure each target at eight compass points so that an average figure would minimise this error. This method should obviate the need for a correction factor (Frost 1976) because of irregular plane of section across a double

label.

The rate of bone formation was measured as the distance between the innermost part of the inner label to the innermost part of the outside label. Frost (1977) has indicated that the measurement should be taken from the middle of each band but this estimation of the middle is subjective. Measurement from the start of one bright band to the start of the next may be a better way of reducing measurement error.

A. Longitudinal sections

Each proximal and distal longitudinal section was divided into four regions for the purpose of measurement, namely graft new (GN), graft old (GO), host new (HN) and host old (HO) (figure IV.2). The area immediately adjacent to the osteotomy site in each of the proximal and distal sections was not measured, as the rate of new bone formation in this area may have been influenced by either the graft or the host. Starting one high power field in from the osteotomy site, each of the four areas was examined in a systematic way for clearly identified double labelled targets. These targets were measured at the eight compass points.

B. Transverse sections

Each transverse section consisted of two areas to be studied:- the graft and its surrounding new bone. Double label targets were located in three high power fields at eight compass points around and within the graft, using the centre of the graft as the axis. Each double label target was then measured on the eight compass points.

Figure IV.1

Photomicrograph of a longitudinal section showing new bone forming around the host bone showing examples of double fluorescent labels. Each "target" (arrow) consists of a brightly labelled inner ring, surrounded by a ring of unlabelled bone and then a further wider band of label. Measurement of the rate of new bone formation was achieved by measuring the distance from the inside edge of the inner label to the inner edge of the outside label using an eyepiece graticule, positioned on each of eight compass points around the target. Microscope magnification was 250, under oil immersion, using ultraviolet lighting conditions.

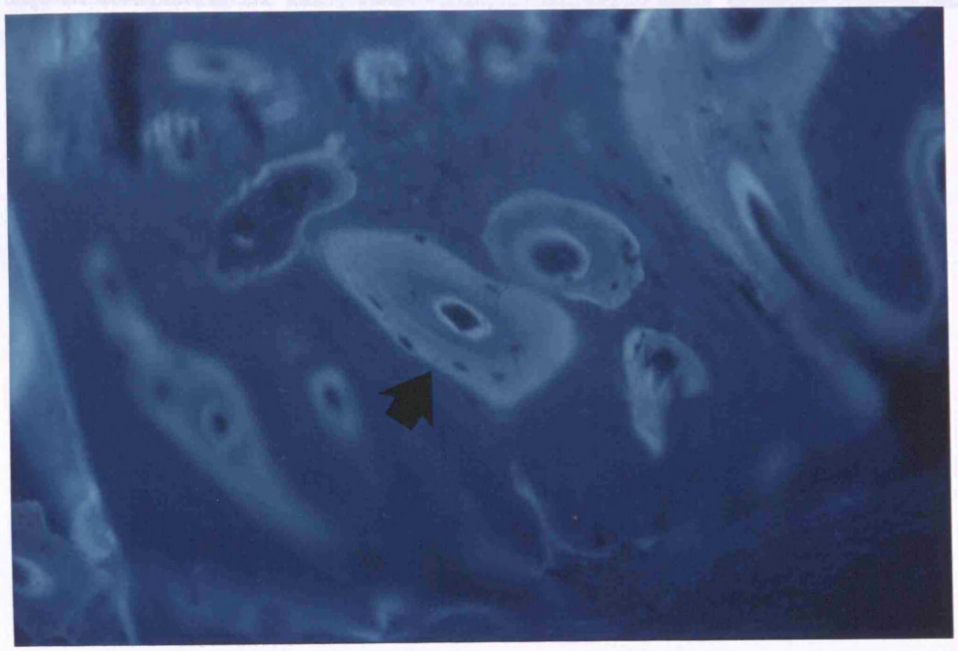
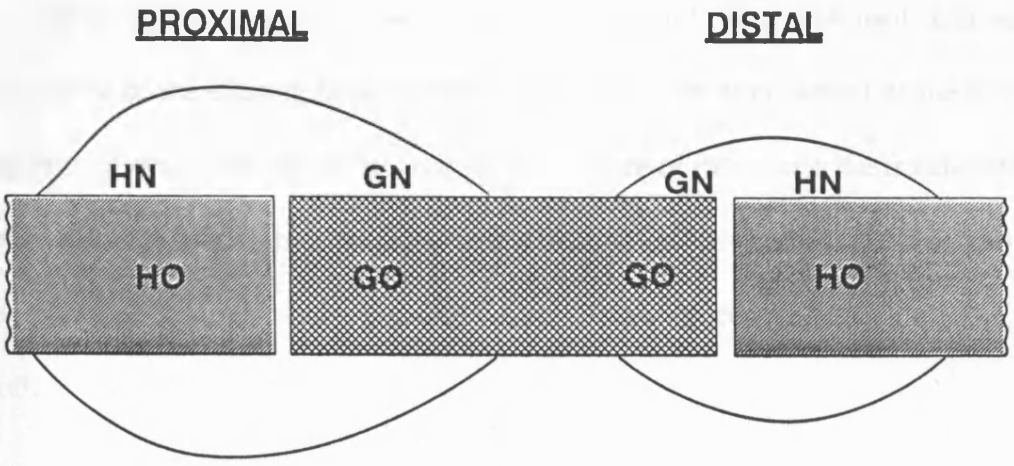


Figure IV.2.

Diagram to show how each longitudinal section provided eight study areas. At each proximal and distal osteotomies there was the new bone around the host HN and the graft GN, and the remodelling in the host HO and the graft GO to be studied.



Method of quantification of fractionated amount of new bone formation

A point counting technique using an eyepiece graticule (Aherne 1982) was used to determine the fraction of the area of the bone occupied by bone labelled with fluorescent label. Using the principle of Delesse the fractionated surface area of labelled bone was then extrapolated to estimate the fractionated volume of labelled bone. This fractionated volume of bone label was deemed to be a reasonable estimate of the amount of the existing bone that was laid down in the time period of the labels having been given. It was therefore an indirect measure of rate, since the fractionated volume of labelled bone is a measure of the amount of new bone laid down in a specified period of time, in this case three weeks from injection of the first label to the cull.

The same section used for measurement of rate of new bone formation was re-examined with a magnification of 125 for measurement of fractionated amount of new bone formation. The amount of new bone formation was measured using a graticule with 36 points by recording hits on labelled and unlabelled bone. The ratio of:-

counts on labelled bone / counts on unlabelled and labelled bone

was used to determine the fractionated amount of new bone laid down in the fixed three week period of bone labelling (from the first injection to the cull).

A. Longitudinal sections.

The same 4 areas (HN,GN,HO,GO) of each section were assessed as above (Fig IV.2). In the areas HN and GN, the graticule was placed in 9 positions of a matrix of 3 neighbouring positions along the longitudinal axis of the bone section and 3 perpendicular to it. The starting position was one low power field in from the osteotomy. In the areas HO and GO, the graticule was placed in 6 positions of a matrix of 3 graticules along the axis of the bone and 2 perpendicular to it. The host and graft cortex was not wide enough to sustain a matrix three graticules wide. The difference in matrix size for HO/GO and HN/GN sections resulted in the data being handled separately for statistical analysis.

B. Transverse sections

Measurement of amount was by three grids of 36 points at low power (x125) at eight compass points in the graft and in the new bone around it.

Statistical methods

The results from both rate and fractionated amount of new bone, as measured by fluorescent microscopy, were entered onto a personal computer statistical program (Minitab Version 8, 1991). The results were analysed by analysis of variance using the Minitab program and therefore the data was entered directly into this program with this in mind.

The data was organised in a dividing hierarchy of cells (or nests) within each level.

For rate of bone formation the hierarchy is as follows:-

3 Groups

4 Animals in each

3 Sections for each animal (proximal longitudinal, distal longitudinal and transverse)

4 areas of study (GO, HO, GN, HN)

15 targets for rate of bone formation

8 compass points on each target

For fractionated amount the hierarchy is as follows:

3 Groups

4 Animals in each

3 Sections for each animal (proximal longitudinal, distal longitudinal and transverse)

5 areas of study (GO, HO, GN, HN and endosteal)

Host new and graft new

3 low power fields depths (close to, middle or furthest away from periosteal surface)

3 low power fields wide (close to, middle and furthest away from the osteotomy)

Host old and graft old

2 depths; periosteal and endosteal

3 low power fields wide (close to, middle and furthest away from the osteotomy)

Endosteal

The data was tested for normality by performing normal scores (Nscores) for each data point. Normal probability plots were then performed for each of the sets of data plotting the Nscores of the data against the data itself. If the sample is from a normal population, then the points usually fall in a straight line whereas if the data is from a non-normal population the plot is curved. The straightness of the plot was measured by the correlation coefficient. If the correlation value is high this is consistent with normality but if low then the hypothesis of normality is rejected. The correlation coefficients were assessed using the table in the Minitab Reference Manual (1991 exhibit 5.1).

If the data proved not to be normal, then a natural logarithmic transformation (Armitage and Berry 1987) was performed before repeating the Nscores and normal probability plots. If the natural logarithmic data was normally distributed, this was used for the analysis of variance. Analysis of variance is a powerful method of analyzing the way in which the mean value of a variable is affected by classification of the data. Most of the analysis was one-way analysis, a generalisation of the unpaired t-test, appropriate for any number of groups.

More complicated analysis of blocks within the data can be handled using the two-way analysis of variance. However, due to some data loss, it was decided that the more forgiving general linear model method of analysis would be a better method since the numbers in each block were unbalanced (Armitage and Berry 1987 and Minitab 1991).

As mentioned above, the data for the fractionated amount of HN/GN and HO/GO areas was collected in a different fashion and therefore this data was separated out for analysis. It was also clear from early histological analysis that the processes of new bone formation outside the host and graft was due to a rather different process than that seen within the host and graft. Interpretation of the double labels seen within the host and graft, and that outside led to the conclusion that the pattern of labelling inside the bone may not match that seen outside the bone (Vide infra page 135). For these additional reasons the rate of new bone formation measurements were handled separately for statistical analysis in the HO/GO and the HN/GN areas.

Results

All twelve animals survived in good general health to the end of the experiment with cull at 8 weeks. Two animals developed an abscess after the second injection of tetracycline which discharged spontaneously. This resulted in these two animals failing to show a second band of tetracycline labelling on subsequent fluorescent microscopy.

Union

A. Clinical union

After cull of each animal, the tibia was stripped of soft tissue. Clinical union at both proximal and distal osteotomy sites was tested directly for rotational stability. The results were recorded as solid union (S), definite fibrous union (F) or possible fibrous union (?).

The results of clinical assessment of union of the osteotomies at cull are shown in table IV.1.

TABLE IV.1

Clinical and radiological estimation of union at the proximal and distal host / graft junctions at cull (8 weeks).

P = proximal and D = distal graft / host junctions. S = solid union, F = fibrous union, ? = indefinite union. G = radiological state of integrity graft; Fract = fracture. No grafts showed evidence of resorption.

Group	Animal	Clinical		Radiological		
	number	P	D	P	D	G
Autograft	1	S	F	F	F	Intact
	2	S	S	S	S	Intact
	3	S	S	S	S	Intact
	4	S	F	S	F	Intact
Allograft	1	S	F	F	?	Intact
	2	S	F	F	F	Intact
	3	S	S	*	*	Intact
	4	F	S	F	F	Intact
Irradiated	1	S	F	?	F	Intact
Allograft	2	?	F	F	F	Intact
	3	F	S	F	?	Fract
	4	F	S	F	S	Fract

* Although both host / graft junctions did not show definite signs of bone union there was a solid bar of bone extending from the proximal to the distal host bone, bypassing the graft.

B. Radiographic union

The final radiographs were examined for evidence of bone union, fracture and resorption (Weiland and Phillips 1984) (table IV.1). In all cases there was a reaction of new bone formation from the ends of the host bone which attempted to encircle or cup the end of the graft. In most cases this did not join onto the graft. The bulk of the new bone was laid down in the posterolateral aspect of the leg (Figure IV.3). The final faxitron radiographs were graded as probable solid union (S) if there was bridging callus between the host and graft (Figure IV.4), fibrous union (F) if there was no or little attempt at bridging callus and possible fibrous union (?) if there was some callus but the osteotomy did not appear to have been fully bridged by callus. The graft was also assessed for evidence of fracture or obvious resorption. In one case there was a solid bar of bone connecting the proximal and distal ends of the host bone but which appeared to by-pass the graft (Figure IV.5). Two grafts sustained spiral fractures and examination of the radiographs taken every two weeks revealed that one occurred between the second and fourth weeks and the other between the fourth and sixth weeks (Figure IV.6). Both belonged to the irradiated allograft group. No grafts showed obvious resorption.

Figure IV.3.

Photograph of Faxitron radiograph of the tibia at eight weeks from implantation of an autograft. The wire sutures have been removed but the intramedullary pin is still in situ. New bone is seen arising from both host ends mainly along the postero-lateral aspect of the leg. The graft is not united to the host proximally or distally. This is animal no. 1 in the autograft group in table IV.1.

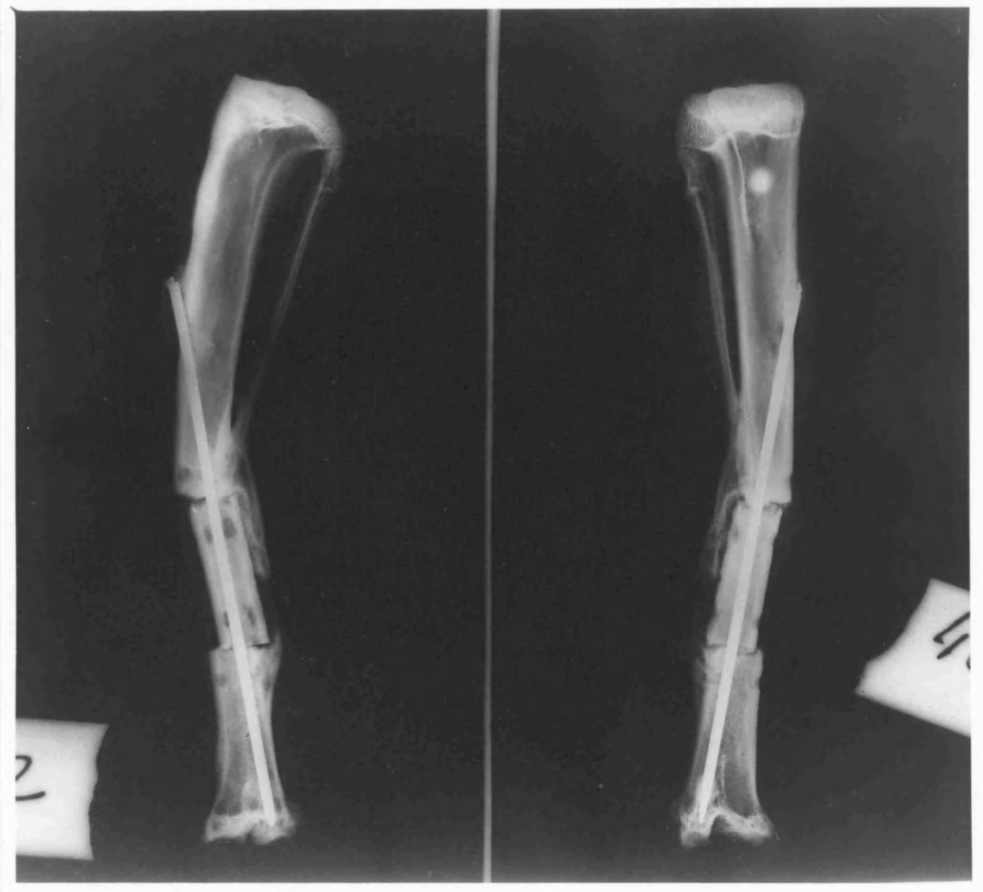


Figure IV.4.

Photograph of Faxitron radiograph of the tibia at eight weeks from implantation of an autograft. Most of the wire sutures have been removed but the intramedullary pin is still in situ. New bone has formed from both ends of the host and united to each end of the graft. This animal is no. 2 in the autograft group in table IV.1.

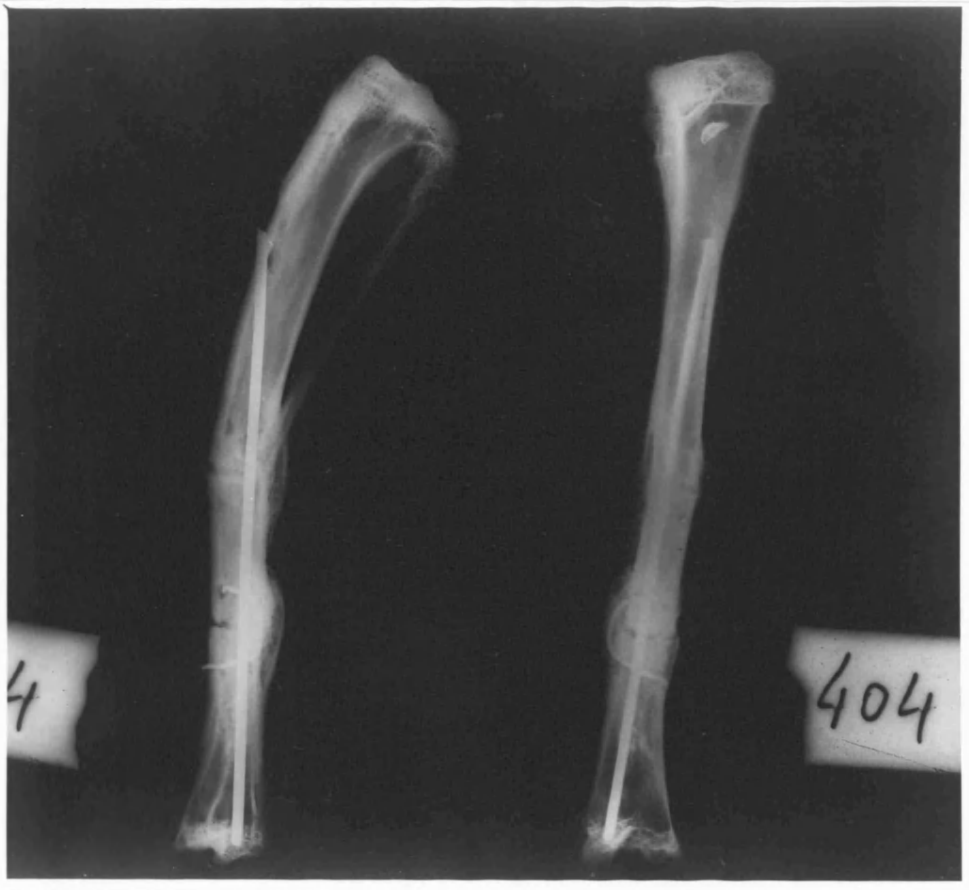


Figure IV.5.

Photograph of Faxitron radiograph of the tibia at eight weeks from implantation of an allograft. The wire sutures have been removed but the intramedullary pin is still in situ. New bone is seen arising from both host ends and has formed a solid bar of bone down the postero-lateral aspect of the leg. Clinically the construct was stable but the radiographs reveal that the graft is not united to the host. This is animal no. 3 in the allograft group in table IV.1.

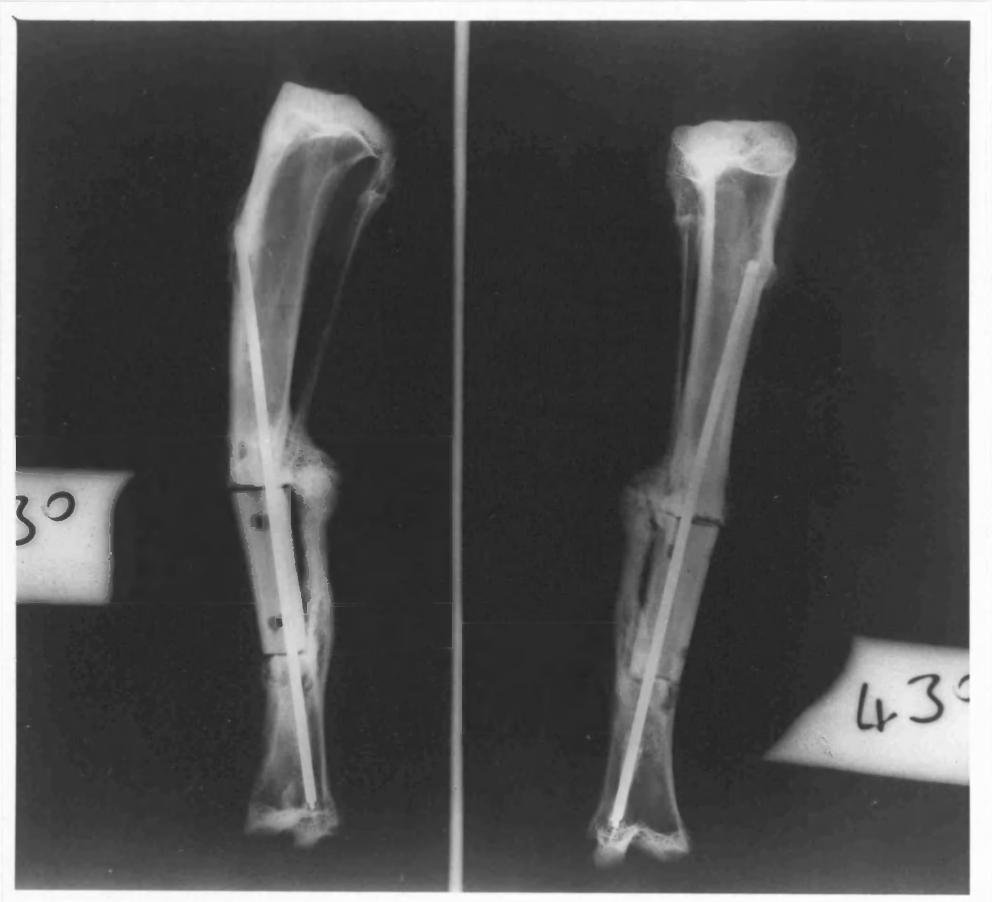
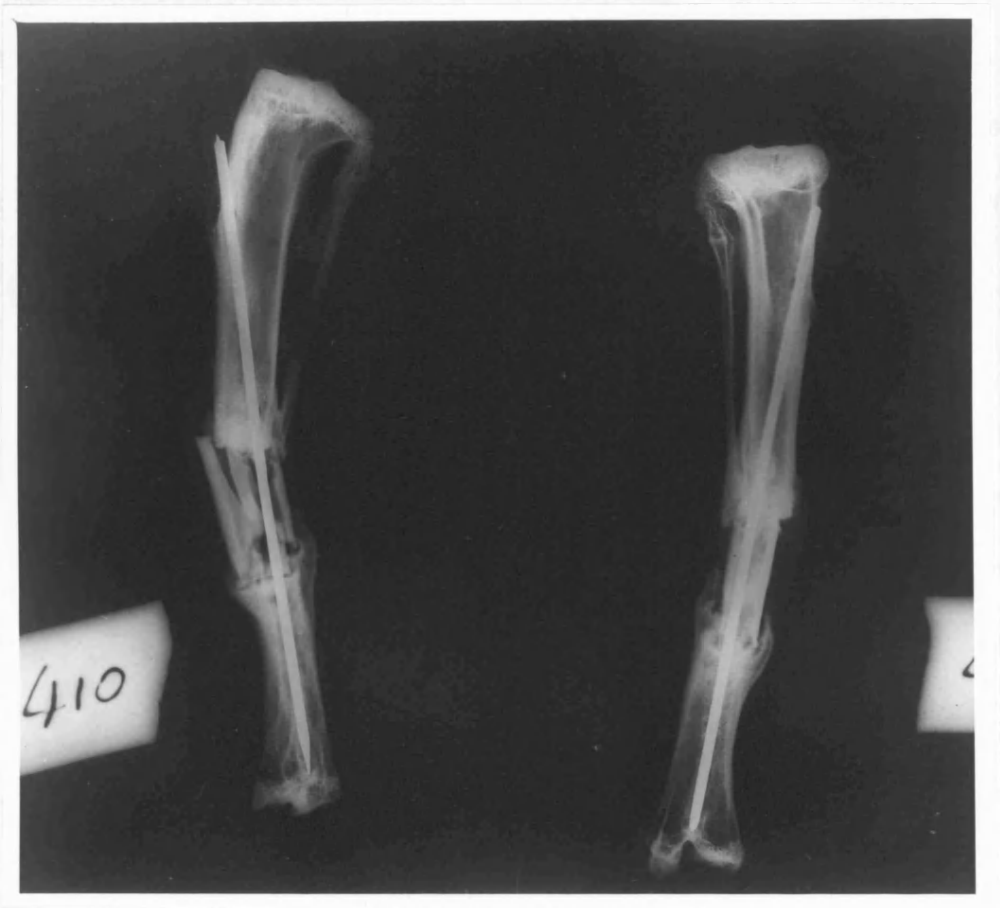


Figure IV.6.

Photograph of Faxitron radiograph of the tibia at eight weeks from implantation of an irradiated allograft. The wire sutures have been removed but the intramedullary pin is still in situ. New bone is seen arising mainly from the distal host end and may be starting to unite to the graft but the osteotomy is still visible. There is a fracture of the graft which occurred between the fourth and sixth weeks. This is animal no. 3 in the irradiated allograft group in table IV.1.



Fluorescent microscopy

Qualitative results of fluorescent microscopy

During the preparation of one of the specimens, there was failure of the embedding process, which resulted in fragmentation of the specimen during cutting of the block (one of the proximal longitudinal specimens of the autograft group). All other specimens processed satisfactorily. In two animals (one of each of the allograft groups) the second label was not absorbed because of abscess formation and therefore only the first label could be identified on fluorescent microscopy. This made the specimens unsuitable for measurement of rate of new bone formation. However, the distribution of the single label in the different areas under study mirrored that seen in those animals which absorbed both labels and therefore these specimens were included in the analyses of the amount of new bone formation.

The identification of double labelled "targets" was easy in the areas of new bone formation around the graft and host bone, enabling measurement of the rate of new bone formation. Each double label consisted of a bright inner band surrounded by an area of unlabelled bone and then a further wider zone of fluorescence (Figure IV.1).

New bone was laid down around both the graft and the host. This new bone appeared to be due to reaction of the periosteum of the remaining host bone, maximal close to the osteotomy sites and fading out along the length of the host bone (figure IV.7). The new bone formed around the ends of the graft. The periosteum was thickened.

There were two clearly identifiable labels under the periosteum related to the mineralisation of the new bone laid down by the periosteum at the times the labels were given. There was extensive remodelling of the host bone with activity in both the endosteal and periosteal surfaces (Figure IV.8). The autograft bone showed good evidence of revascularisation with cutting cones and new bone formation (Figures IV.9 and IV.10). This was rarely seen in the allograft bone (Figure IV.11).

Figure IV.7

Photomicrograph of a longitudinal section showing the edge of the host bone away from the osteotomy site, with the periosteal reaction along the length of the host bone. The thickened periosteum (arrow) is seen outside the double label of new bone. Microscope magnification was 250, under oil immersion, using ultraviolet lighting conditions.

Figure IV.8

Photomicrograph of a longitudinal section of an area of host bone close to the osteotomy. Intensive remodelling with labelled new bone formation can be seen throughout the cortical bone. Note the area of unlabelled bone (arrow) seen within a band of fluorescent label. Microscope magnification was 250, under oil immersion, using ultraviolet lighting conditions.

Figure IV.9

Photomicrograph of a longitudinal section of an area of autograft bone away from the osteotomy. There is abundant new labelled bone outside the graft. A fluorescent labelled cutting cone (arrow) is seen invading the graft. Microscope magnification was 125, using ultraviolet lighting conditions.

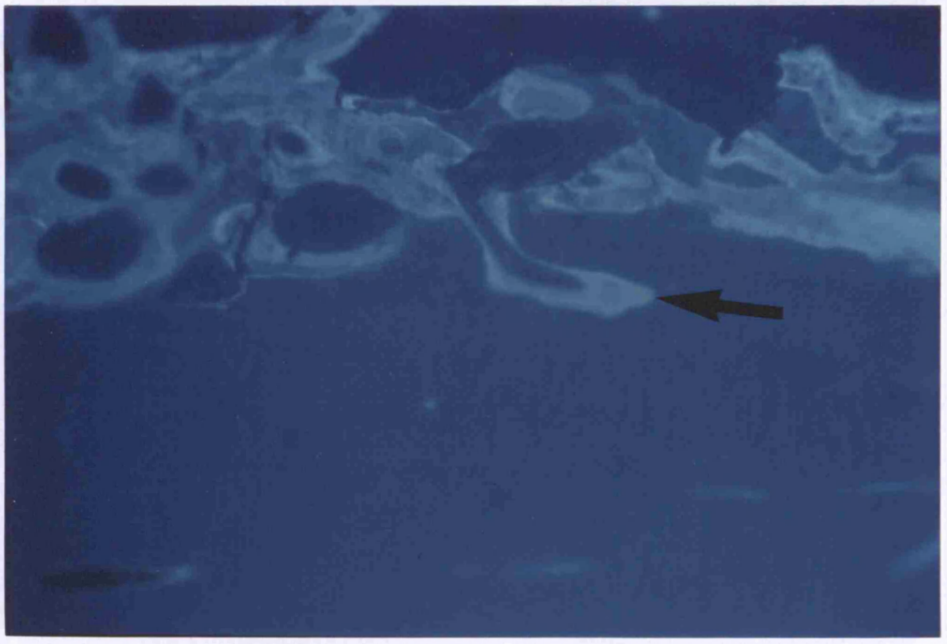


Figure IV.10

Photomicrograph of a longitudinal section of an area of autograft bone at the osteotomy (arrow). Intensive remodelling with labelled new bone formation can be seen throughout the autograft cortical bone. Compare to figure IV.11. Microscope magnification was 250, under oil immersion, using ultraviolet lighting conditions.

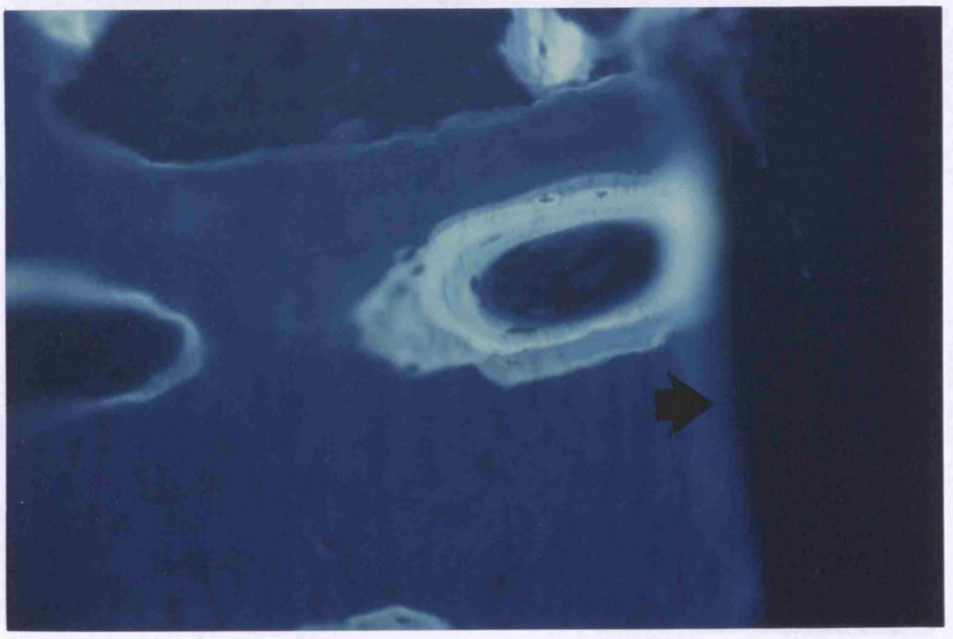


Figure IV.11

Photomicrograph of a longitudinal section of an area of allograft bone at the osteotomy (bold arrow). There is minimal remodelling of the graft, only occurring at the periphery (open arrow). Compare to Figure IV.10. Microscope magnification was 125, using ultraviolet lighting conditions.

Fluorescent microscopy

The quantitative data

The data for rate and amount of new bone formation was tested for normality by converting the data into normal scores using Minitab. A normal probability plot of the data against the normal scores was then undertaken. The straightness of the distribution was then assessed by doing a correlation coefficient. A powerful test of normality, which is essentially similar to the Shapiro-Wilk test, can be based on this correlation. A very high coefficient is consistent with normality and the hypothesis of normality was rejected if the coefficient fell well below the critical value in the tables (Minitab Reference manual). The results are shown in table IV.2.

With the exception of transverse sections and the amount of new bone formation in the host bone (HO) and graft (GO), all the other data was normal or near normally distributed. The data for the amount of new bone within the graft and host was biased by the large number of zero values, skewing the data to the left. By natural logarithmic transformation this data could then be handled as normally distributed (Correlation coefficient 0.984). Although not all of the data reached true normality by the Shapiro Wilks test, it was close enough to use it in the robust analysis of variance. The data for both rate and amount in the transverse sections did not fit a normal distribution due to the large number of zero values. There was an obvious difference between the groups and therefore analysis of variance was performed on the raw data.

Table IV.2

Test of normality of data sets by use of Nscore and correlation coefficients.

SECTIONS	DATA	CORRELATION COEFFICIENT	NORMALITY
LONGITUDINAL	Rate	0.991	yes
	Amount HNGN	0.993	yes
	Amount HOGO	0.953	no*
	Amount Endosteal	0.986	nearly
TRANSVERSE	Rate	0.954	no
	Amount	0.954	no

* Natural logarithmic transformation of this data for HOGO produced a near normally distributed data set (0.984) which was used for further analysis.

In each of the study areas, the analyses were done using oneway analysis of variance, the steps of which can be outlined as follows (see Appendix 3). The between-groups and the within-groups sums of squared deviations from the mean (SS) are calculated. Each of these values is then divided by the relevant degrees of freedom (DF) to calculate a mean square (MS) for each. The between-groups means square is divided by the within groups means square to yield the variance ratio (F score). If this value is sufficiently high, i.e. of low probability (P), then it is statistically significant. The relevant Minitab program demands that the probability should be zero or virtually zero to be statistically significant. Each of the tables in appendix 3 show the calculation of the F Score. They also show a visual analogue of the mean for each variable and the 95% confidence intervals around the mean using the pooled data.

Results of rate of new bone formation

The quantitative results for rate of new bone formation in longitudinal sections are shown in tables IV.3 and IV.4. The values are the measured distance between the two labels in the targets in each area of study. The units are arbitrary. N = number of double labelled targets measured. The number of observations was lower in the allograft groups because of failure of one animal in each of the allograft groups to take up the second label. The number of observations in the proximal osteotomy group was less than the distal group because one proximal block fragmented on cutting for sections. The data was normally distributed allowing analysis of variance. The data for new bone around the graft and host (table IV.3) was analysed separately from that of the revascularisation within the graft and host bone (table IV.4) for the reasons given on page 135.

A. Rate of new bone formation around the host and graft (HN & GN areas).

See Table IV.3 and appendix 3.

The highest rate of new bone formation was seen in the autograft group which was significantly more than in either of the two allograft groups which were similar. There was no significant difference between the proximal and distal osteotomy. However, there was a significant difference in the rate of new bone formation in the different areas studied. The fastest rate was seen in the area around the host (HN) and the slowest in the area around the graft (GN). This was due to the periosteal response around the host bone. The periosteum around the graft had been removed in creation of the 2 cm defect and therefore the response of the periosteum was confined to the

host area. However, the periosteal response around the host cupped the bone graft and so new bone was available for measurement around the ends of the graft.

B. Rate of new bone within the host and graft (HO & GO).

See Table IV.4 and appendix 3.

The results were the same as those of the rate of new bone formation outside the graft and host bone. There was a significant difference between autograft and allograft but not between the two allograft groups. There was no significant difference between the osteotomies but the rate of new bone formation was greater within the host (HO) in comparison to the graft (GO). This is not surprising in view of the fact that the graft was avascular at the time of the re-implantation, unlike the vascular host bone.

Table IV.3

Summary of results of rate of new bone formation around the host (HN) and graft (GN). Values are the mean and standard deviations of measurements of the distance between two labels (arbitrary units). Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables and analogues.

		Number of measurements	Mean	Standard deviation	P =
Type of graft	Autograft	210	8.3	4.7	0.0
	Irradiated allograft	180	3.0	3.9	
	Allograft	180	2.1	2.7	
Osteotomy	Proximal	270	4.6	4.9	0.69
	Distal	300	4.7	4.8	
Area of section	Host new	285	6.7	4.3	0.0
	Graft new	285	2.6	4.4	

Table IV.4

Summary of results of rate of new bone formation within the host (HO) and graft (GO). Values are the mean and standard deviations of the measurements of the distance between two labels (arbitrary units). Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		Number of measurements	Mean	Standard deviation	P =
Type of graft	Autograft	210	2.7	4.0	0.0
	Irradiated allograft	180	1.7	2.9	
	Allograft	180	1.2	2.1	
Osteotomy	Proximal	270	2.1	3.3	0.20
	Distal	300	1.8	3.1	
Area of section	Host old	285	3.6	3.5	0.0
	Graft old	285	0.23	1.4	

Fractionated amount of new bone formation.

The data for the fractionated amount of new bone in the old graft and host areas (GO HO) was collected in a different way to that of the new bone around the graft and host bone (GN HN) and was therefore analysed separately (page 86).

A. Fractionated amount of new bone formation around the graft and host (GN & HN). See Table IV.5 and appendix 3.

The results of the fractionated amount of new bone laid down around the host (HN) and graft (GN) in longitudinal sections are shown in table IV.5a. This data was normally distributed allowing analysis of variance. The means and standard deviations were based on the ratio of:-

hits on labelled / hits on labelled + unlabelled bone.

The amount of new bone was significantly higher in the autograft group in comparison to the allograft groups. There was no significant difference between the two allograft groups. However the distal osteotomy showed significantly more new bone than the proximal. This is surprising in view of the more extensive muscle attachment to the tibia more proximally. The new bone around the host (HN) was more plentiful than around the graft (GN) which matches the improved periosteal response. There was a significant relationship between the amount of new bone and the distance from the osteotomy site, more new bone being seen closer to the osteotomy and gradually lessening away from the osteotomy.

The general linear statistical model was used to assess if the difference between the autograft and allograft groups was related to the host or graft Table IV.5b. There was a significant difference between the autograft and allograft groups in the new bone around the graft. This was less true of the new bone around the host bone (the periosteal response), suggesting that the main difference between the groups was related to the new bone around the graft.

B. Fractionated amount of new bone formation in the graft old and host old areas (HO & GO). See Table IV.6 and appendix 3.

The results of the fractionated amount of new bone laid down in the revascularisation of the host (HO) and graft (GO) in longitudinal sections are shown in table IV.6. The data for fractionated amount of new bone formation in the Host old HO and Graft old GO areas was not normally distributed but natural logarithmic conversion produced data which was normally distributed, allowing analysis of variance. The means and standard deviations are of the natural logarithm of the ratio:-

hits on labelled / hits on labelled + unlabelled bone.

The fractionated amount of new bone laid down, as revascularisation proceeds, in the autograft group was significantly more than in the two allograft groups. There was no significant difference between the proximal and distal osteotomies. The host bone (HO) showed significantly more new bone laid down than within the graft (GO) which is likely to be related to the better vascularity of the host bone compared with the graft. There was no significant difference in the measurement of the periosteal and

endosteal areas of cortex. There appeared to be no relationship between the amount of new bone laid down within the cortex of the graft or host bone and the proximity to the osteotomy site unlike that of new bone laid down outside the host and graft bone which did vary with the distance from the osteotomy (table IV.5).

C. Fractionated amount of new bone formation in the endosteal areas.

See Table IV.7 and appendix 3.

The results of the amount of new bone laid down in the endosteal area of the bone in longitudinal sections are shown in table IV.7. The data was near normally distributed. Means and standard deviations are based on the ratio of:-

hits on labelled / hits on labelled + unlabelled bone.

The amount of new bone in the endosteal region did not vary between the three groups of animals. There was no significant difference between proximal and distal osteotomies. There was significantly more new bone in the endosteal area of the host than in the graft. This may explain why no difference was found between the autograft and allograft groups, as the majority of the new endosteal bone was seen within the host, not within the graft and is therefore unlikely to vary with the type of graft.

Table IV.5a. Summary of results of amount of new bone formation around the host (HN) and graft (GN). Values are the mean and standard deviations of N measurements of the labelled / (labelled and unlabelled) counts. Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		N	Mean	Std Dev	P=
Type of graft	Autograft	126	0.55	0.21	0.0
	Irradiated allograft	144	0.38	0.28	
	Allograft	144	0.28	0.30	
Osteotomy	Proximal	198	0.35	0.30	0.004
	Distal	216	0.44	0.28	
Area of section	Host new	207	0.46	0.19	0.00
	Graft new	207	0.33	0.36	
Proximity to the osteotomy	Close	138	0.45	0.28	0.008
	Middle	138	0.40	0.29	
	Away	138	0.34	0.30	

Table IV.5b. Results of the general linear model used to determine if the difference between the autograft and allograft groups was due to the new bone around the host (HN) or the graft (GN). The results are the mean and standard deviations of the fractionated amount measurements. The P value was 0.00. See appendix 3.

Graft	New bone around host	New bone around graft
Autograft	0.52 (0.03)	0.59 (0.03)
Irradiated allograft	0.46 (0.03)	0.29 (0.03)
Allograft	0.42 (0.03)	0.14 (0.03)

Table IV.6. Summary of results of amount of new bone formation within the host (HO) and graft (GO). Values are the mean and standard deviations of N measurements of the natural log [labelled / (labelled and unlabelled)] counts, hence the minus values. Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		N	Mean	Std Dev	P =
Graft	Autograft	63	-2.5	0.66	0.001
	Irradiated allograft	51	-2.1	0.74	
	Allograft	52	-2.0	0.89	
Osteotomy	Proximal	79	-2.3	0.78	0.54
	Distal	87	-2.2	0.80	
Area	Host old	130	-2.0	0.66	0.00
	Graft old	36	-3.0	0.75	
Area	Periosteal	84	-2.2	0.80	0.70
	Endosteal	82	-2.2	0.79	
Proximity to osteotomy	Close	61	-2.3	0.84	0.28
	Middle	56	-2.1	0.75	
	Away	49	-2.2	0.78	

Table IV.7. Summary of results of amount of new bone formation in the endosteal regions of the host and graft. Values are the mean and standard deviations of N measurements of labelled / (labelled and unlabelled) counts. Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		N	Mean	Std Dev	p=
Graft	Autograft	90	0.48	0.41	0.41
	Irradiated allograft	105	0.46	0.41	
	Allograft	83	0.40	0.43	
Osteotomy	Proximal	133	0.49	0.39	0.14
	Distal	145	0.41	0.43	
Area	Host	158	0.64	0.33	0.00
	Graft	120	0.20	0.37	

D. Transverse sections. See tables IV.8 and IV.9 and appendix 3.

The transverse sections from the middle of the graft tended to fragment on cutting, especially in the tissues surrounding the graft. The GO area was therefore the only area studied.

The results of the rate of new bone laid down in revascularisation of the graft (GO) in the transverse sections are shown in table IV.8. Means and standard deviations are of the distance between the two labels in the targets. The units are arbitrary. The data were not normally distributed but was subjected to a oneway analysis of variance without transformation to reinforce the qualitative impression.

The results of the fractionated amount of new bone laid down in revascularisation of the graft (GO) in the transverse sections are shown in table IV.9. Means and standard deviations based on the ratio of:-

hits on labelled / hits on labelled + unlabelled bone.

The data was not normally distributed but analyses are by oneway analysis of variance.

Tables IV.8 and IV.9 show that the results for rate and fractionated amount of new bone formation were similar. New bone formation was rarely seen within both types of allograft but was seen in autograft.

Table IV.8. Summary of results of rate of new bone formation in the graft in the transverse sections. Values are the mean and standard deviations of N measurements of the distance between two labels in the same arbitrary units. Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		N	Mean	Std Dev	p=
Graft	Autograft	45	7.6	3.1	0.0
	Irradiated Allograft	45	0.0	0.0	
	Allograft	45	0.0	0.0	

Table IV.9. Summary of results of amount of new bone formation in the graft in the transverse sections. Values are the mean and standard deviations of N measurements of labelled / (labelled + unlabelled) counts. Analysis by oneway analysis of variance with p value shown. See appendix 3 for the more complete tables.

		N	Mean	Std Dev	p=
Graft	Autograft	96	0.26	0.34	0.0
	Irradiated Allograft	96	0.05	0.17	
	Allograft	96	0.08	0.25	

Haematoxylin and Eosin staining.

The H&E specimens generally processed well and were less liable to fragment than the fluorescent specimens particularly in the transverse sections.

These sections confirmed the impression gained from the fluorescent work that the new bone around the host (HN) and graft (GN) was due to periosteal reaction, maximal close to the osteotomy (Figure IV.12). Remodelling of the host bone (HO) was seen with aggressive activity in both the endosteal and periosteal surfaces (Figure IV.13).

The transverse sections from the middle of the graft revealed that the autograft bone was usually united to surrounding new bone. There was intense activity on both the endosteal and periosteal surfaces of the graft with marked revascularisation (Figure IV.14). In contrast, new vessel formation was only seen close to the surface of the allograft bone in the transverse sections (Figure IV.15) and rarely was there new bone united to the middle of the allograft where the transverse sections had been cut. There was no major difference in the appearance of the two allograft groups.

The longitudinal sections revealed that the autograft tended to unite at the osteotomy sites with new bone emanating from both the periosteal and endosteal surfaces of the host bone (Figure IV.16). The allograft specimens revealed that while the host bone response was similar, the new bone did not unite to the allograft well but rather tended to encase it (Figure IV.17). The extent of revascularisation of the allograft was low and superficial (Figure IV.18). There appeared to be little difference between the

two allograft groups. The was little difference between the proximal and distal osteotomies.

Figure IV.12

Photomicrograph of a longitudinal section of host bone close to the osteotomy. The periosteum (P) can be seen as an artefactually separated thickened layer. New bone has been laid down joined to the host bone along a noticeable cement line (C). More new bone has be laid down close to the osteotomy site (O), gradually lessening further from the osteotomy. Many nuclei are seen within the cortex of the host suggestive of remodelling, particularly on the periosteal and endosteal aspects but less marked in the middle of the cortex. A cutting cone (CC) is seen in the endosteal area of the cortex. Compare to Figure IV.7. Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.

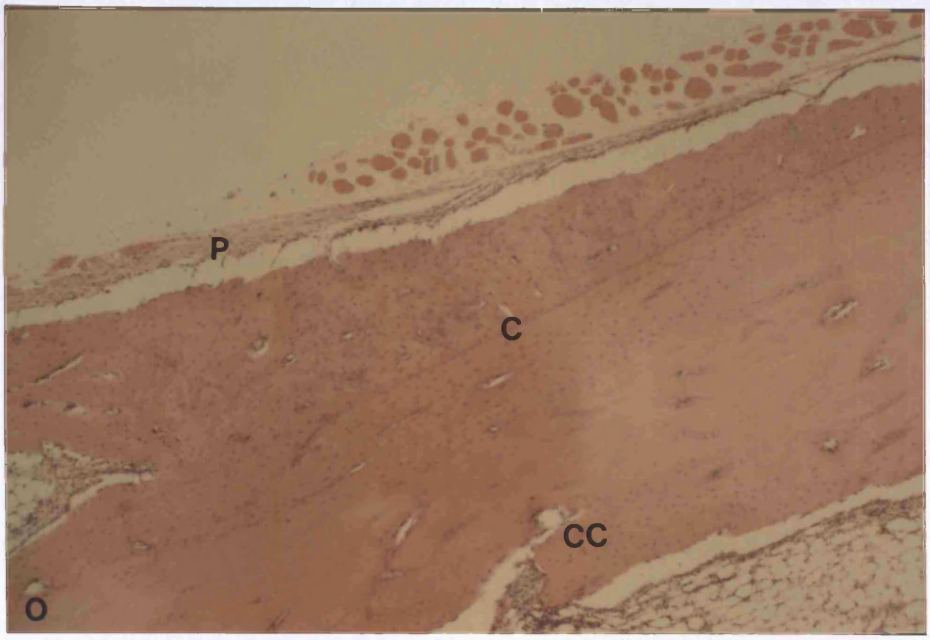


Figure IV.13

Photomicrograph of a longitudinal section of host bone. New bone can be seen on the periosteal (P) and endosteal (E) surfaces. There is intense cellular activity within the host, particularly in the area adjacent to the periosteum. Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.

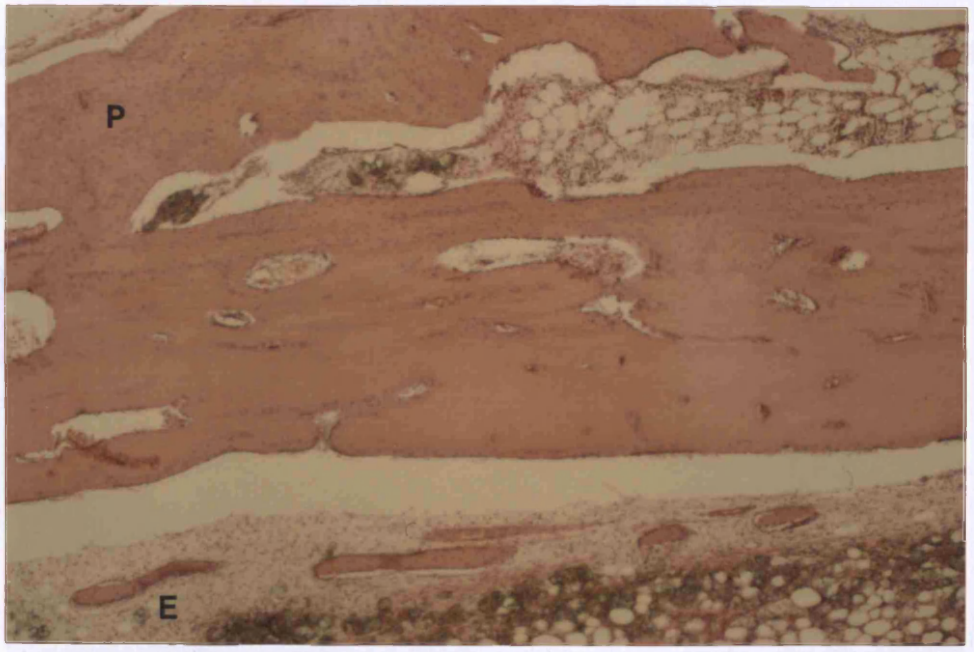


Figure IV.14

Photomicrograph of a transverse section of the middle of an autograft. New bone can be seen on the outer surface which is united to the graft along a cement line. There is no periosteum which was removed at the surgery. There is cellular activity throughout the graft which appears to have completely revascularised. The medullary canal (E) was cleared at the time of surgery and has become filled with new bone, osteoid and vascular spaces. The new bone in the medulla appears to have united to the endosteal surface of the graft. Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.

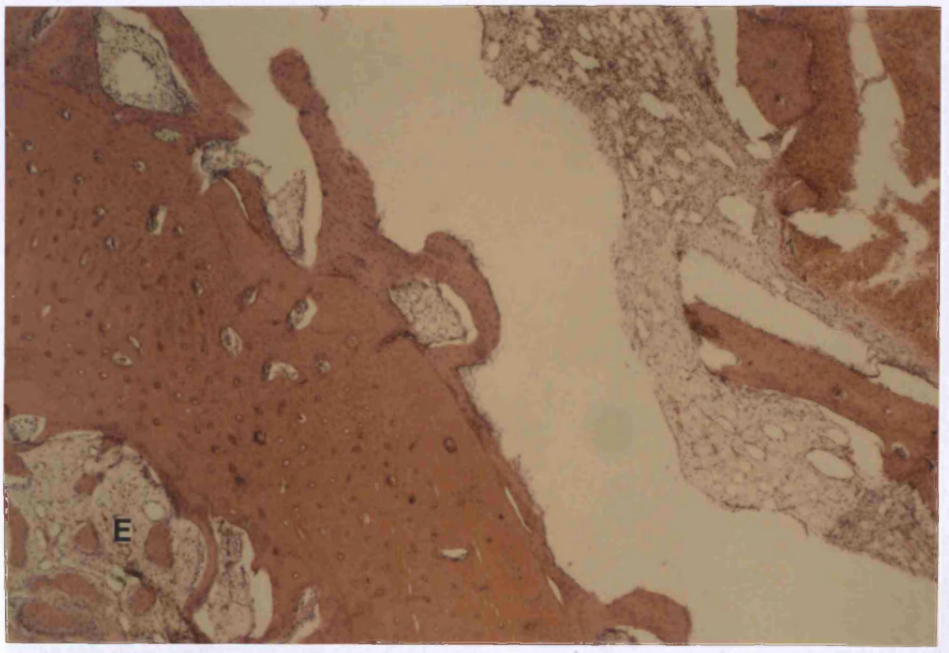


Figure IV.15

Photomicrograph of a transverse section of the middle of an allograft. Osteoid and some new bone can be seen on the outer surface but there does not appear to be union of the new bone to the graft. There is some invasion of the outer surface of the graft (arrow) but very few cell nuclei are seen within the bone lacunae. The majority of the graft appears inert. In the medullary cavity there is evidence of fibrous stroma with some osteoid but no mature bone. The appearances are strikingly different to those of an equivalent section of autograft; compare to figure IV.14. Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.

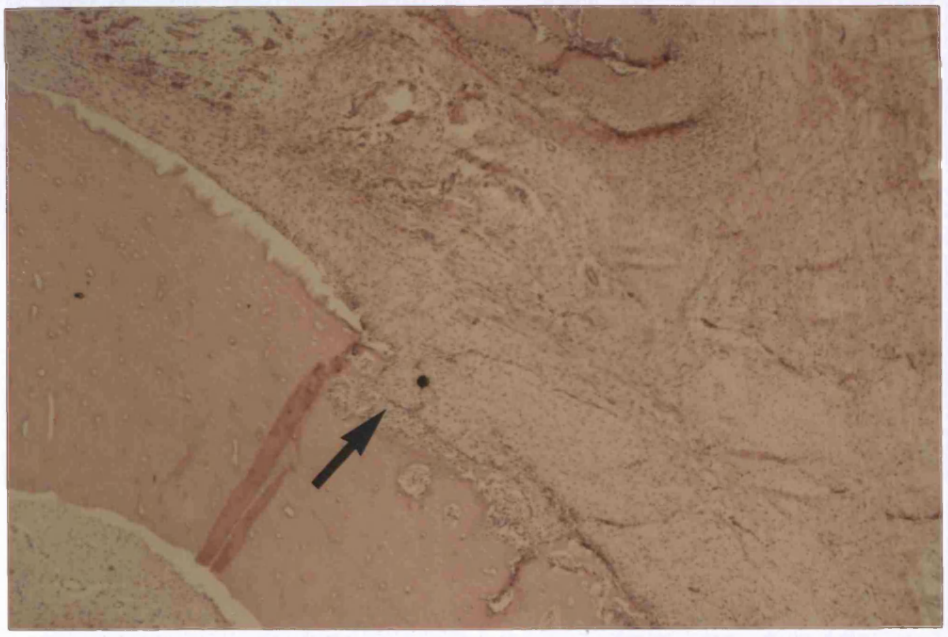


Figure IV.17

Photomicrograph of longitudinal sections of an irradiated allograft (G) at the osteotomy. There is a prolific amount of new bone extending from the periosteal surface (P) of the host extending around the graft. Fibrous stroma and early osteoid are seen in the osteotomy gap. There is a fibrous reaction in the endosteal canal (E). There is no union of the new bone to the graft which appears inert. There is considerable remodelling activity in the host bone (H). Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.

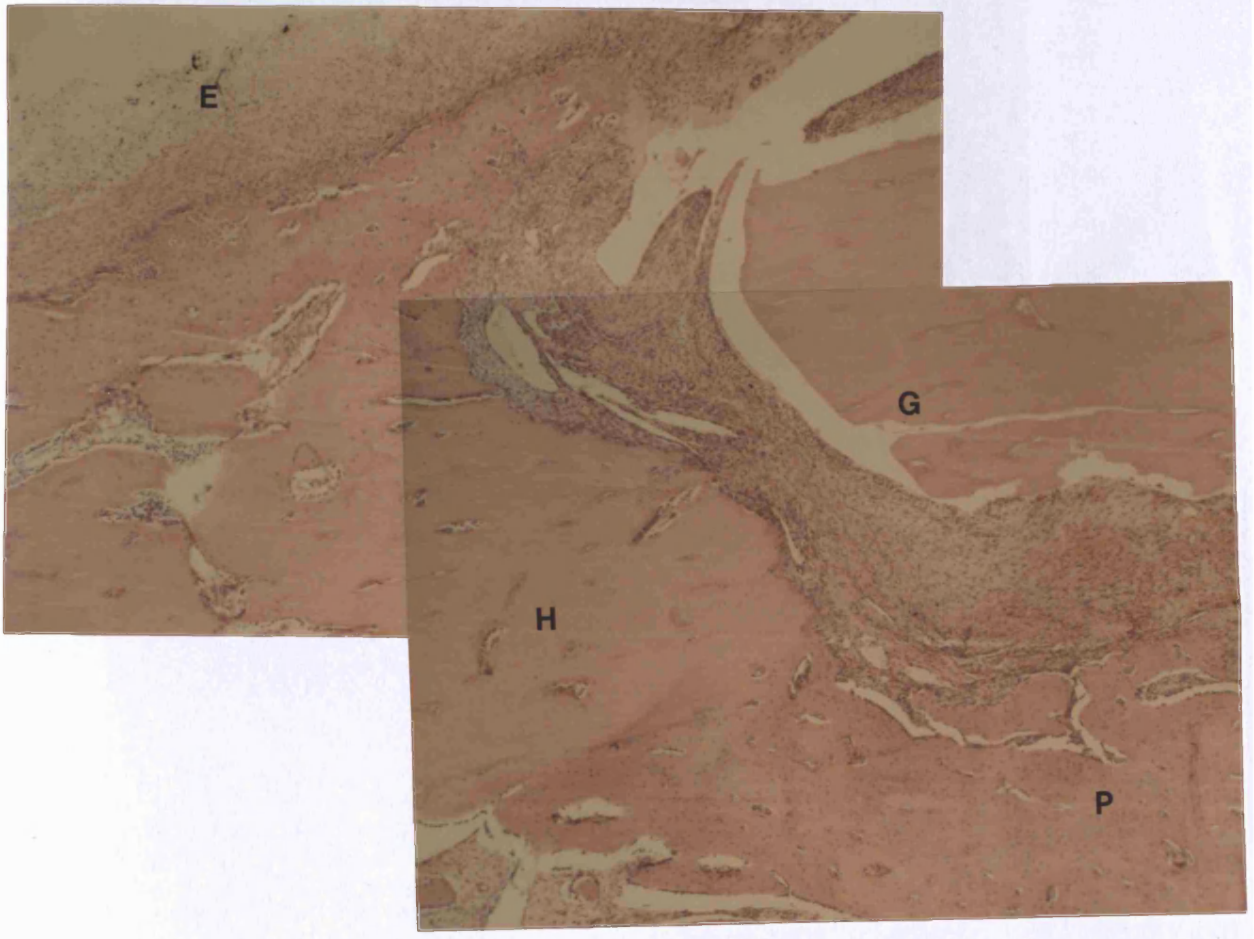
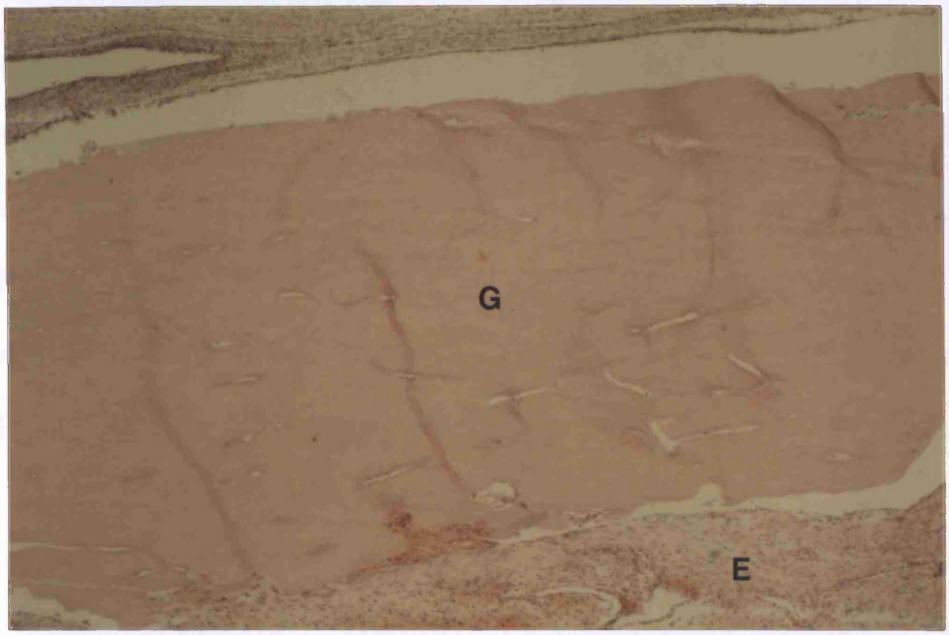


Figure IV.18

Photomicrograph of a longitudinal section of an irradiated allograft (G) close to the osteotomy. Some fibrous stroma and early osteoid is seen within the medullary canal (E). The graft is relatively inert with empty lacunae. There is a little early invasion of the graft with new vessels particulary on the endosteal side. Haematoxylin and Eosin stain. Microscope magnification was 40, using standard lighting conditions.



Assessment of Errors

There were many potential sources of error in this experiment. The sample size was very small with only four animals in each of three groups. For each animal, a number of slices from blocks were prepared but only one representative slide from each was subject to measurement. However, each slide was examined twice, once for rate measurements and once for fractionated amount of new bone formation. The results from these two types of measurement were in agreement.

A small error study was performed to assess the repeatability of the rate measurements. One section was examined on two different days; one hundred measurements of rate were made on each occasion. The results in table IV.10 show that no significant difference was observed between the results using a Students "t" test. The method was repeatable with a low intra-observer error, probably due to the large number of measurements made on each slide.

The measurement of rate of new bone formation depended on the identification of two bands of fluorescence. The method in which the rate measurements were obtained was found subsequently to have a potential error and is discussed more fully on page 135. The fractionated amount of new bone formation was measured using an eyepiece grid of 36 points placed repeatedly in a systematic way on the subject. This systematic pattern of measurement was used to reduce the likelihood of sampling error.

Table IV.10

Results of distances between two labels on the same slide when measured on two occasions.

Measure	Sample Number	Mean	Std Dev	STD Error	95% C. I.
1	100	8.40	3.09	0.31	7.79-9.00
2	100	8.35	3.42	0.34	7.68-9.02

Discussion

Previous authors, most notably Buring and Urist (1967) and Weintroub and Reddi (1988), have evaluated the effect of irradiation on the potential for bone graft to induce new bone formation. Their work was done on lower animals as in this experiment. The new bone formed was quantified by transplanting the graft, usually decalcified, into an ectopic site so that the confounding influence of the host bone and surrounding structures, such as the periosteum, had been removed. The results obtained by Urist and Weintroub are at odds. In addition, the very placement of the graft into an ectopic site removes some of the potentially essential requirements for new bone formation. The assumption that Urist has made, with regard to induction of new bone, is that the inducing factors are derived from the graft because of its ability to induce new bone in an ectopic site. However, what is not known or represented in his experiments, is the possible interaction of the inducing agents of the graft and the host structures around the bone defect or their effect on the remodelling process of the graft.

This animal study showed that the periosteum around the remaining host bone reacted in a most powerful way to the injury of the operation and removal of the segment of tibia. The pattern of reaction was similar to that observed in other rabbit experiments on fracture healing (Oni 1987). The response was maximal at the fracture site, fading gradually, the further from the fracture site that the observation was made. It was not known what effect the bone graft might have on this periosteal response. The periosteal response might be independent of the graft material, purely determined by

the local biological and mechanical environment, after creation of the defect and subsequent reconstruction. It is also possible that the properties of the graft, similar to those that Urist observed in his ectopic transplantation experiments, may have a predictable effect on the periosteal response, the response of the endosteum or the soft tissues around the bone. Finally, there is the possibility that the graft may have interacted with the host bone, periosteum and surrounding muscles in a manner not previously observed.

There was a similar but less pronounced effect seen within the endosteal side of the bone. Most of the new bone formed appeared to be derived from the ends of the host bone, through either a periosteal or endosteal response. There appeared to be no spontaneous bone formation from the soft tissues themselves around the middle of the graft. All of the new bone that surrounded and bonded to the graft "grew" from the host ends. This questions the relevance of the bone induction experiments, using graft in an ectopic muscle site, in relation to the incorporation of a bulk allograft. It may be that the vigorous periosteal response masks any subtle effect on osteoinduction produced by the different type of allografts. However, the pattern of incorporation in this experiment mirrors that seen in human radiological studies of massive allograft incorporation (Loty et al 1990, Hernigou et al 1986). It is possible that the work of Urist may be of more relevance to the use of morsellised graft used in situations where the host periosteum has a limited influence, rather than in structural, bulk allografts.

It is also necessary to consider the revascularisation, remodelling and new bone

formation within the graft. Johnson and Stein (1988) noted that there appears to be a difference between autograft and allograft in this process of creeping substitution (Phemister 1914). Cortical autograft appears to remodel by revascularisation of existing channels within the Haversian systems and internal renovation of existing osteons in an extensive but orderly fashion. In contrast, cortical allograft has a slower and more limited replacement of cortical bone, primarily at the proximal and distal host-graft interfaces and proceeding towards the centre. This was observed in this experiment (see Fig IV.14 & Fig IV.15).

It was hoped that the removal of the periosteum around the excised segment would ensure that it played no further role in the experiment. Although every effort was made to ensure complete removal of the periosteum, it is possible that a few fragments of the periosteum around the bone defect may have remained. It was also clear from the early experiments (page 69) that the fibula had a powerful influence on union of the graft, which was hopefully, but not certainly, removed in the main study.

The method of fixation did not provide sufficient stability to dispense with external support. In consequence, the animals did not make full use of the injured limb and were observed to be not fully weight bearing which may have had an influence on the bone response to the graft. However, the same conditions existed for all three groups of animals.

Double fluorescent labelled targets were easily identified in the areas of new bone

formation around the graft and host bone. The predominant pattern of each double label consisted of a bright inner band surrounded by an area of unlabelled bone and then a further wider zone of fluorescence. In some instances it was possible to identify a ring of unlabelled bone surrounded by a double label. Because cull took place one week after the administration of the second label, it was assumed that any new bone laid down in the last week before cull would bear evidence of the label. The unlabelled bone that was observed within the double label was therefore considered to have been laid down before the bone that was labelled. It was thus assumed that mineralisation took place from within outwards and that the inner label represented the new bone mineralised after administration of the first label and that the outer label likewise following administration of the second label. The outer label was generally wider in appearance, less clearly defined and less brilliant than the inner label. It is not clear why the outer label should be wider. One possibility is that the speed of new bone formation increases with the increasing size of the new bone seam. The reduction in brilliance may be due to a dilutent effect on the label because it is being laid down over a much longer circumference.

It was considered reasonable to estimate rate of bone formation as the distance between the innermost part of the inner label to the innermost part of the outside label. Frost (1977) has indicated that the measurement should be taken from the middle of each band but it may be difficult to identify the middle of a zone of fluorescence leading to subjective errors. Measuring the distance from the start of one bright band to the start of the next may be the best way of reducing this measurement error and was the method chosen in this experiment. However this

method may not be appropriate for new bone formation within the graft or host.

Double labels were also seen in the revascularisation process of the graft and host but unlabelled new bone was not seen within double labelled rings. However, unlabelled new bone was seen within one label only. The same method of quantification was used as in the rate of new bone formation in the new bone around the graft and host i.e. from the inside of the inner label to the inside of the outer label. However, work from other authors (Johnson and Stein 1988) suggests that the deposition of mineral in the remodelling of cortical bone occurs from outside to in, within each remodelling Haversian canal. Thus, the outer label in this situation may represent the first label and the inner label, the second label. This may be different to the situation outside the graft and host bone. The measurement method undertaken throughout was that from the inside of the first (outer) label to the inside of the second (inner) label. As a consequence, the data for rate of measurement should be viewed taking this into account with respect to the graft and host old bone. For this reason and because the deposition of new bone outside the graft and host (bone induction) is by a different process to that observed inside the graft and host (bone conduction), the data was separated for the purposes of the statistical analyses. One way of confirming the temporal pattern of mineralisation would be to use differently coloured labels at different times.

Loss of study animals

The study group consisted of twelve animals but two animals failed to take up the second label because of abscess formation at the site of injection. Histological study

of these two animals showed that the pattern of deposition of the first label was identical to that observed in other animals. It was therefore decided to include the fractionated amount of new bone measurements in the analysis. It was accepted that their inclusion would bias the fractionated amount results against the allograft groups. However, the fractionated amount results supported the results of the rate of new bone formation.

One of the specimens from the autograft group fragmented on cutting, prohibiting measurement of either rate or fractionated amount of new bone. This specimen was therefore excluded from the analysis. Analysis of the rest of the specimens from that animal showed that there was nothing peculiar to that animal, with a deposition of the label in the same manner as the other autograft animals. It was felt that this exclusion would not seriously bias the results of the autograft group.

The histomorphometric methods used

One of the purposes of this project was to explore the use of tetracycline labelling to measure the rate of bone formation around and within bone graft. Tetracycline binds reliably to sites of active bone mineralisation (Milch 1958, Villaneuva 1983). The assumption made in this project is that the rate of mineralisation is a reasonable measure of the rate of bone formation. It is assumed that the rate of mineralisation of osteoid tissue is constant and therefore, the uptake of tetracycline label during mineralisation reflects the rate of production of the osteoid tissue. Other studies have validated tetracycline labelling as a measure of the rate of bone growth. The preceding discussion highlights the practical difficulty in using the distance between

two tetracycline labels as a measure of the rate of bone formation. The labels were hard to interpret from a temporal perspective and therefore the measurement points may be inaccurate. Use of the recommendations of Frost (1977) that the middle of each label be the points of measurement would have resulted in more reliable data on the rate of bone formation in the revascularisation process of the host and graft. If this double label method is to be used, it is now recommended that the measurements are made from the middle of each label and that labels of different colours are used so that the temporal sequence is obvious (Johnson and Stein 1988, Stevenson et al 1991). Clearly, these multiple labels must be visible under the same ultraviolet lighting conditions, if rate measurements are to be made. A simple pilot error study suggested that the method of measurement of rate of new bone formation used here is reproducible. However, the method was laborious and time consuming.

The method of measurement of fractionated amount of new bone formation used in this project allows estimation of what proportion of bone visible has been laid down in the labelling periods. Labels were given at three weeks and one week prior to cull of the animal. Thus, in the two week interval, some unlabelled bone may have been laid down during the last three weeks of life of the animal. Thus the labelled bone represents some, but not all, of the bone laid down in the last three weeks of life. However, the same conditions applied to each animal (with the exception of the two that failed to take up the second label). Therefore, for comparative purposes, the measurement of fractionated amount of new bone formation allows estimation of the proportion of new bone laid down in the last three weeks.

For the areas outside the graft and host it is therefore a measure of the proportion of new bone formation in the last three weeks i.e. a measure of rate of new bone formation. However, within the host and graft bone, it is a measure of the proportion of the existing bone that has undergone remodelling with new bone in the last three weeks. For this measurement, only one label is necessary. The point counting method is a time honoured method of measurement based on the Delesse principle. It is however time consuming, and future studies should use an automated area measurement device. The use of two or more different coloured labels would allow measurements of the fractionated amount of new bone at different times, giving a more dynamic picture of bone remodelling (Stevenson et al 1991, Johnson and Stein 1988).

Evaluation of the results

The radiographic evidence pointed to strong influence of the periosteum in the union of the graft to the host. Both the proximal and distal host bone reacted with cupping of the graft and subsequent union to the graft. The early experiments had shown that the fibula was also involved in this process, but placement of the osteotomy below the syndesmosis removed this influence. The one case of a solid bar of bone laid down parallel to the graft suggested that some of the periosteum of the removed segment was inadvertently left behind when the defect was created.

Radiographic scoring systems devised by Goldberg (1989), Weiland and Phillips (1984), Lane and Sandhu (1987) were not easy to apply. No cases of resorption were seen but there were two fractures, both in the irradiated allograft group. This arouses

suspicion that the irradiation may have structurally weakened the graft but the study was not specifically designed to test this. Judgement of union was difficult and very subjective. An attempt at quantification of volume of bone on the radiographs was made but was crude and inconclusive. Magnification of the radiographs by digital imaging and quantification might make this approach more useful.

The previous discussion has revealed questions about the reliability of some of the data on rate of new bone formation as measured by the two label technique. However, even with this proviso, what did the data from this experiment reveal?

New bone around the host and graft; the external callus

The results suggest that there is a difference between the new bone laid down around autograft and allograft. This was confirmed on both rate and fractionated amount measurements and is a reassuring result, in agreement with most studies which suggest the superiority of autograft. (Kirkeby et al 1992, Heiple et al 1963, Tagaki and Urist 1982). Further analysis suggests that this difference is mainly due to the different amount of new bone around the grafts, the new bone around the host being similar in all three groups. Thus, it appears that the periosteal reaction is the same in the three types of grafts but that the extension of this response to the tissues around the autograft is superior to that in the two allograft groups. Heiple et al (1963) found only a slight delay in new bone formation around allografts compared to autografts at four weeks but the effect became more pronounced by three months.

The model is sensitive enough to demonstrate a difference in the type of graft that is generally accepted, i.e. autograft versus allograft. However, there appeared to be no difference between the two allograft groups and therefore the null hypothesis in Chapter I (page 42) was not excluded. This may be because there is no real difference between the allograft groups. The model may be insufficiently sensitive to detect a difference although one exists, or there are insufficient numbers used to demonstrate a difference.

Both the rate and fractionated amount measurements suggested that the new bone was formed more rapidly around the host, rather than around the graft. This is to be expected in view of the fact that there is no periosteum around the graft. The same pattern of new bone formation was seen on qualitative microscopy i.e. new bone forming at the host osteotomy site which cupped the graft until union took place. Analysis of the fractionated amount of new bone suggests that the maximum activity outside the bone was closest to the osteotomy site and that this gradually faded the further one sampled away from that site in both the graft and host. Thus the osteotomy influenced the activity of the periosteal response. It is of note that other experiments, where more rigid internal fixation has been used, much less external callus and more internal callus was formed, leading to invasion of each end of the graft (Johnson and Stein 1988, Stevenson et al 1991).

The proximal osteotomy site is more deeply buried within muscle and it might have been presumed that this could lead to increased surrounding blood supply and possibly to increased new bone formation in the soft tissues. Furthermore, the nutrient and

accessory nutrient arteries were likely to have been injured in the operation (Morgan 1959). The analysis of the rate results showed that there was no difference between the proximal and distal osteotomy sites, but the fractionated amount suggested that the distal osteotomy was associated with more new bone. This may because of a time delay at the proximal and distal sites. The proximal site may be more advanced and therefore not laying down as much new bone as the distal by the time the labels were given. Johnson and Stein (1988) found a tendency for faster proximal union than distal but their model had more rigid fixation with less external callus.

New bone within the host and graft cortical bone; creeping substitution

The remodelling activity within the bone may be less dependant on the periosteum. The activity within the host can be looked upon as a remodelling process in response to the injury. In the graft however, the remodelling is a function of the revascularisation, the bone resorption and new bone formation within the graft. This should model the osteoconductive properties of the graft (Buchardt 1983). Both the rate and fractionated amount results demonstrated a difference between the autograft and allograft groups but not between the two allograft groups. This closely matches the results outside the bone. Thus, revascularisation of autograft is superior to that of allograft. This is consistent with the findings of many other authors (Heiple et al 1963, Johnson and Stein 1988, Stevenson et al 1991). It is possible that the activity outside the bone determines the revascularisation of the graft and host bone and that this accounts for the difference, but the work of Johnson and Stein (1988) suggests that this applies even when there is no external callus. They are of the opinion that there is a marked difference in the pattern of revascularisation of allograft and

autograft. They suggest that part of this difference may be as a result of the immune response to the allograft.

In this experiment, the rate and fractionated amount of new bone found within the host was significantly more than that in the graft. However, there was no difference at the proximal and distal osteotomy sites suggesting that the possible difference in the environments of these two osteotomy sites was not sufficiently important to influence the rate of revascularisation. No difference was found between the fractionated amount of new bone in the endosteal surface and the periosteal surfaces of the remodelling cortical graft and host bone. Other authors have noted that the substitution of allografts tends to be more peripheral (periosteal) (Stevenson et al 1991).

The transverse sections taken from the middle of the graft showed no double labels within the allografts at this level, suggesting that the middle of the graft is the last piece to be revascularised. The autograft did however have double labels in the middle of the graft showing the superior revascularisation of this graft as did Johnson and Stein (1988). The sections demonstrated creeping substitution of the graft from each end but this was not supported by the quantitative analysis, perhaps because the sampling method only looked at the area of bone close to the osteotomy.

New bone within the medullary canal; the endosteal response

Analysis of the endosteal new bone in the medullary canals showed a significant difference between graft and host but not between the types of graft. This might

suggest that the difference between allografts and autografts is not related to revascularisation of the empty medullary canal.

Conclusions

Both the fluorescent and H&E sections produced a similar picture. The large amount of new bone produced around the host was secondary to a periosteal reaction. This periosteal response was very similar to that seen in fracture healing models (Oni 1987) and in other structural allograft models (Nather et al 1990). In addition there was intense activity seen in the endosteal part of the host bone close to the osteotomy. The new bone appeared to grow around the graft cupping it and eventually uniting to the outer surface and its cut surface, agreeing with the findings of Nather et al (1990). The direction of growth of new bone from the host producing the cupping appeared to be determined by the graft. In addition the rate and amount of new bone cupping the graft is determined by the type of graft, autograft performing better than either of the allograft groups. Revascularisation of the graft appeared to take place from both ends and from both the periosteal and endosteal surfaces near the osteotomy sites. Revascularisation was more advanced in the autograft specimens. There was no evidence in this experiment that irradiation of the allografts impaired their ability to induce new bone in the surrounding tissues or in the revascularisation of the graft. The null hypothesis therefore could not be rejected.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Introduction

Extrapolation of any results of lower animal experiments to the human situation is hazardous. Nevertheless, study of incorporation of bone graft in human is difficult. Not often afforded the luxury of graft retrieval, histological study of bone graft incorporation in patients is rarely possible. Removal of graft because of infection or graft failure due to fracture or non-union may provide some information but is biased towards those grafts which fail. Some autopsy studies (Gie et al 1993, Nellison et al 1995 and Enneking 1991) do provide histological information on graft incorporation but provide only a snap-shot in a lengthy process.

It would be desirable to study human bone graft incorporation by non-invasive investigation. Radiographs may show evidence of graft healing, revascularisation and remodelling (Gie 1993, Nellison 1995, Enneking 1991). However, they are perhaps ideally suited to show failure of graft incorporation, remodelling or fracture and may not be sufficiently sensitive to study graft incorporation. The use of radiographs in animal experiments has been coupled to scoring systems to assess healing (Goldberg 1989, Lane and Sandhu 1987, Weiland and Phillips 1984) but the findings of the present study suggest that this method is probably best reserved for crude assessment. Radioactive bone scans have been used in human and animal work but are not completely reliable (Stevenson et al 1974, McMaster and Merrick 1980). The use of newer imaging techniques, such as magnetic resonance imaging, may provide valuable

information in the future.

Animal models provide a possible solution to the problem of study of bone graft incorporation by allowing autopsy histological studies. However, it is well recognised that animal bone may behave differently to human bone and this is particularly true of lower animals such as rodents, rabbits etc. It is therefore imperative that the researcher, who uses animal models, must be cautious about extrapolation of the results. This project was planned to investigate the manner in which large bone grafts heal by labelling the new bone that had formed and to assess the effect, if any, of irradiation of the bone allograft on that healing process.

Bone graft incorporation

The quantitative and qualitative histology results give rise to a model of bone graft incorporation. The surgery and defect appear to induce a periosteal reaction of the host bone, maximal at the osteotomy site, gradually fading further from this along the host bone. There is also activation of the endosteal host bone. The cells in the host cortical bone close to the osteotomy die following the surgery and remodelling of the host cortical bone follows later. The periosteal new bone forming around the host spreads out into the haematoma and soft tissues around the graft.

This new bone appears to receive some signal to tell it grow around the graft and try to unite to it. This signal may be from the graft itself or possibly in response to movement, similar to the response of a fracture. The fact that autograft and allograft

produce differing amounts of new bone around the graft, but the same amount around the host bone, implies that the type of graft may have an influence on this process. Thus, it is possible that local factors produced by the graft, e.g BMP, interleukins, growth factors etc, account for the difference in the new bone around the graft but not around the host bone. This model of external callus formed by the host bone has more of the features of the theory of Barth than those of Baschkirew and Petrow (Page 10). The osteogenic potency of the graft (Urist and McLean 1952) appears to involve the periosteal response of the host, albeit there is certainly a possibility that the graft induces new bone from the surrounding tissues. Of interest, is the finding of Nather et al (1990) that encasing the bone graft in a silastic sheath, to prevent contact with surrounding muscle, caused a significant delay in union of the periosteal response to the graft when compared to controls. Similar isolation of the medullary canal of the graft caused only a slight delay. It would thus appear that the periosteal response is the most critical factor for graft / host union but this may well be modulated by the graft.

Revascularisation and remodelling of the graft appears to take place from each end and from both the endosteal and periosteal surfaces of the graft near the osteotomy sites (Buchardt 1983). The creeping substitution is not as a result of surviving cells suggested by Phemister (1914) but rather from vascular invasion from without. This process was more rapid for the autograft than for the allograft (Johnson and Stein 1988, Stevenson et al 1991). The difference is most likely to be due to immunological consequences of the graft on the revascularisation process.

In this model, there appeared to be no difference between the irradiated and non-irradiated allograft in either the new bone formed around the graft or in the revascularisation of the graft. The findings are more in keeping with those of Weintroub and Reddi (1988) than of Buring and Urist (1967). The purpose behind this project was to investigate the potential of a holistic type of model to study the incorporation of the graft, rather than provide the definitive answer as to whether irradiation of the graft is detrimental to its incorporation.

Future Research

The small sample size of rabbits and limitations of the method make extrapolation of this data to the human situation difficult. It does suggest however, that the holistic approach taken in this experiment may be important for future work. The interaction between the graft and the response of the host tissues is critical. The use of demineralised bone matrix in an ectopic site removes from study the influence of the host bone. A standard model would help all researchers to test the hypotheses in a systematic way. Suggestions for improvement in the model include the following:-

1. Use of a higher animal whose bone structure and development more closely matches that of the human.
2. A more thorough attempt to remove all of the host periosteum around the defect i.e. a true tumour operation.
3. A more rigid method of internal fixation which allows full weight-bearing e.g. plating or locking nail fixation.

4. Use of different coloured sequential fluorescent bone labels to accurately map new bone formation.
5. Cull at different intervals to give a more complete picture of new bone formation and revascularisation. Following this, a "standard" time could be used to test the influence of the method of graft preparation.
6. Use of fractionated amount of new bone formation by automated counters rather than rate of bone formation as the method of quantification.
7. The possible use of magnetic resonance imaging to monitor graft remodelling in vivo.

There are many different facets to this problem; the biomechanical, ultrastructural, immunological, union and revascularisation / remodelling aspects. Although it is important to separate different facets of this process, one must not lose sight of the goal of a united, functioning structural allograft in the clinical situation. All of the facets mentioned, depend upon the relationship between the host and the graft and these relationships change with time as the graft incorporates and remodels. The use of such a model which mimics the clinical situation, allows manipulation of the interaction and can quantitate the effect of this manipulation, may provide further understanding of the process of graft incorporation.

Similar research could be combined with biomechanical, immunological, cell culture studies to provide a more complete understanding of bone graft incorporation and what factors influence it. If the assertion that 2.5 Mrads is not sufficient to sterilise bone completely is true, then previous work may have to be repeated using higher

doses of irradiation. The literature suggests that higher doses may have effects on the strength and the ability of bone to integrate successfully. If this is the case, then alternative methods of preparation of the bone graft may have to be followed. The possibility of culturing the recipient's osteoblasts, combining these with osteoinductive factors and a structural scaffold, starts to look very attractive indeed (Nolan et al 1992).

CONCLUSIONS

The literature and the data in this review, suggest that there is a very real problem with local infection following the use of structural allografts in the reconstruction of large bone defects. It has been supposed that contamination of the graft may be one of the contributing factors for this but the literature does not tend to support this assertion. Transplant of allogenic material carries with it the risk of disease transmission including the HIV virus. One method of sterilisation of the bone allograft involves irradiation. The generally accepted dose required for decontamination and sterilisation is 2.5 Mrads but more recent research has questioned if this is sufficient to destroy HIV virus deep within bone.

The use of irradiation to sterilise bone may be associated with ultrastructural defects and biomechanical impairment particularly at high doses. At 2.5 Mrads, the literature suggests that the graft should not be impaired from a structural viewpoint. However, there may be a detrimental effect of irradiation at this dosage on the incorporation of the bone graft.

The integration of a large structural allograft depends on two related processes; that of union and that of revascularisation / remodelling of the cortical graft. Union may be dependent upon the osteoinductive properties of the graft, related to inducing factors produced by the graft. Although some studies suggested that irradiation may impair the performance of these factors, these experiments have been performed in isolation from the host bone. It is not clear from these experiments how the inducing

agents influence the process of union and revascularisation / remodelling.

The present experiment was designed to model the clinical situation of the use of a structural allograft and to quantify the integration of the graft. This model was then used to investigate the effect of 2.5 Mrads on the allograft used. The model was successful but improvements for future work have been identified. No obvious effect of irradiation on the integration of the allograft was identified.

APPENDIX 1

Preparation of specimens for Haematoxylin and Eosin staining

Fixation / Embedding

1. The specimens were fixed in 10% neutral buffered formalin for one week without change.

2. They were decalcified in 150 mls Kristianson's solution (900 mls formic acid; 175 Grams sodium formate; 4100 mls distilled water) for one week; the solution being changed every alternate day.

3. Using a tissue processor (Shandon) the following regime was then undertaken:-

4 hrs in 70% alcohol

4 hrs in 90% alcohol

4 periods of 4 hrs in 100% alcohol

3 periods of 4 hrs in chloroform

4 hrs in hot wax

4 hrs in hot wax under vacuum of 0.8 atmospheres

The specimens were then embedded in wax using a hot wax bath.

Cutting

The paraffin blocks were cooled in ice to facilitate cutting on a base sledge microtome (Anglia Scientific) with the knife set at 6 microns and angle of blade at 12°. The block was lined up parallel to the blade and trimmed until tissue was exposed. After cooling in ice again, a ribbon of sections was cut at 6 microns while blowing on block. Sections from middle of the ribbon were teased apart on a bench and placed on a glass microscope slide. The sections were wet with 10% alcohol (helps to transfer to water bath) and then transferred to water bath at 50°C (any higher and wax melts) using the glass slide. The sections were then transferred from free floating in the water bath to a slide, precoated with silane. Blotting paper was placed on top of the slide and weighted to dry flat. After incubation at 37°C overnight, the blotting paper was peeled off and the section was ready for staining.

Staining

The slides were stained using the following regime:-

(1) 2 minutes in xylol

(2) 1 minutes in fresh solution of xylol

(3) 1 minute in Industrial pure alcohol

(4) 1 minute in fresh solution of Industrial pure alcohol

(5) 1 minute in 95% alcohol

(6) rinse in tap water

(7) 4 minutes in Mayer's haematoxylin

(8) rinse in tap water

(9) dip in 1% acid alcohol (70% alcohol, 1% hydrochloric acid)

(10) wash in tap water (2 minutes)

(11) 20 seconds in 0.5% eosin

(12) rinse in tap water

(13) 1 minute in 95% alcohol

(14) 1 minute in Industrial pure alcohol

(15) 1 minute in fresh solution of Industrial pure alcohol

(16) 1 minute in xylol

(17) 1 minute in fresh solution of xylol

Following drying, cover slips were applied to the sections using DPX mountant.

APPENDIX 2

Preparation of specimens for fluorescent microscopy.

Fixing and Embedding

Preparation took place according to the process described by Mawhinney and Ellis 1983 with slight modification.

1. The tissue was placed in glass universal bottles and fixed immediately in methylated spirit (Industrial 74 O.P., Fisons) for 4 days, followed by one day in absolute ethanol (Analytical Reagent, Fisons), at room temperature.

2. The tissue was then impregnated with Polymaster resin for 24 hours at room temperature on a blood mixer (Spiramixer, Denley). This was repeated a further three times with fresh resin.

3. The tissue was further impregnated with 95% resin and 5% Di-n-butyl phthalate (Fisons) for 24 hours at 37° centigrade. This was repeated with two further mixes of fresh solutions. The samples were in loosely capped universal bottles and kept under a vacuum of -0.8 atmosphere in an anaerobic jar (Don Whitley Scientific Ltd).

4. Further infiltration took place with 93% resin, 5% Di-n-butyl phthlalate, 1% Butanone Peroxide solution (Butanox 50, Akzo Chemie UK Ltd) as catalyst and 1% inhibitor (1% Hydroquinone, Specified Laboratory Reagent, Fisons, in absolute ethanol, Analytical Reagent). This was carried out on a blood mixer for 8 hours to

prevent polymerisation of the resin.

5. The specimens in the mixture used in step 4, were transferred to glass jars, previously treated with Dimethyldichlorosilane solution (General Purpose Reagent, BDH) to prevent the specimens sticking to the glass and positioned in the centre of the base of the glass jar. These were transferred to a waterbath at 37° centigrade for 40 hours during which time the resin set. The waterbath dissipates heat from the exothermic reaction as the resin polymerises and avoids bubble formation in the specimen.

6. The resin was further hardened in an oven at 60° centigrade for 48 hours.

7. The glass jars were broken to release the blocks which were trimmed with the band saw to produce parallel sides with 2-3mm resin around the edges of the tissue. The cutting edge of the block was filed to expose the tissue.

Cutting

The cutting face of the block was trimmed in 10 micron steps using a K3 steel knife on a Jung K microtome. An HK2 tungsten carbide knife was used to cut the sections at 7 micron thickness. The block, knife and sections were well lubricated with 70% ethanol. The sections were cut with tissue paper (Izal Medicated Toilet Tissue, Jeyes Ltd, UK) applied with the rough side to the block, to prevent curling.

Mounting

The sections were dried carefully between two pieces of filter paper and removed from the tissue paper using forceps. After further blotting, the sections were transferred to a xylene bath from which they were mounted onto a glass slide. If the sections were too large for the cover slip or an edge had folded, the section was trimmed with a scalpel while on the glass slide. Cover slips were applied with DPX mountant.

APPENDIX 3

Statistical tables.

The following analyses have been carried out on a personal computer using Minitab version 8. Analyses are by one way analysis of variance and the general linear statistical model. See page 106 for discussion of the method. Each table number refers to the corresponding summary tables contained within chapter IV.

STATISTICAL TABLE IV.3

ANALYSIS OF VARIANCE OF RATE OF NEW BONE FORMATION AROUND THE HOST AND GRAFT.

Values are the distances between the two fluorescent labels.

Table IV.3.a. BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	4506.1	2253.0	147.25	0.000
Within groups	567	8675.3	15.3		
Total	569	13181.4			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	210	8.306	4.716	(--*-)
IR Allo	180	3.006	3.904	(--*--)
Allo	180	2.054	2.693	(--*--)
POOLED STDEV =		3.912		2.0 4.0 6.0 8.0

(Auto = autograft; IR Allo = irradiated allograft; Allo = allograft)

Table IV.3b BETWEEN PROXIMAL AND DISTAL OSTEOTOMIES

	DF	SS	MS	F	p
Between groups	1	3.7	3.7	0.16	0.690
Within groups	568	13177.7	23.2		
Total	569	13181.4			

OSTEOTOMY	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Proximal	270	4.573	4.883	(----------*----------)
Distal	300	4.735	4.756	(----------*----------)
POOLED STDEV =		4.817		4.20 4.55 4.90 5.25

Table IV.3c. BETWEEN AREAS ON THE SECTION (NEW BONE AROUND HOST OR GRAFT)

	DF	SS	MS	F	p
Between groups	1	2391.8	2391.8	125.92	0.000
Within groups	568	10789.5	19.0		
Total	569	13181.4			

AREA	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
HN	285	6.707	4.335	(---*--)
GN	285	2.610	4.382	(--*---)
POOLED STDEV =		4.358		3.0 4.5 6.0 7.5

(HN = new bone around host; GN = new bone around graft)

Table IV.4

ANALYSIS OF VARIANCE OF RATE OF NEW BONE FORMATION WITHIN THE HOST AND GRAFT.

Values are the distances between the two fluorescent labels.

Table IV.4.a. BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	218.63	109.31	11.09	0.000
Within groups	567	5588.19	9.86		
Total	569	5806.82			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	210	2.707	3.959	(-----*-----)
IR Allo	180	1.727	2.889	(------*-----)
Allo	180	1.244	2.139	(------*-----)
POOLED STDEV =		3.139		1.40 2.10 2.80

(Auto = autograft; IR Allo = irradiated allograft; Allo = allograft)

Table IV.4b BETWEEN PROXIMAL AND DISTAL OSTEOTOMIES

	DF	SS	MS	F	p
Between groups	1	16.7	16.7	1.63	0.202
Within groups	568	5790.2	10.2		
Total	569	5806.8			

OSTEOTOMY	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Proximal	270	2.116	3.314	(----------*----------)
Distal	300	1.773	3.080	(----------*----------)
POOLED STDEV =		3.193		1.50 1.80 2.10 2.40

Table IV.4c BETWEEN AREAS ON THE SECTION (NEW BONE WITHIN HOST OR GRAFT)

	DF	SS	MS	F	p
Between groups	1	1657.52	1657.52	226.90	0.000
Within groups	568	4149.30	7.31		
Total	569	5806.82			

AREA	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
HO	285	3.641	3.522	(-*--)
GO	285	0.230	1.486	(--*--)
POOLED STDEV =		2.703		0.0 1.2 2.4 3.6

(HO = new bone within host; GO = new bone within graft)

TABLE IV.5

ANALYSIS OF VARIANCE OF FRACTIONATED AMOUNT OF NEW BONE AROUND THE HOST AND GRAFT

Values are:-

LABELLED / (LABELLED + UNLABELLED) counts.

Table IV.5a BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	5.1595	2.5797	34.99	0.000
Within groups	411	30.3003	0.0737		
Total	413	35.4597			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	126	0.5517	0.2140	(----*----)
IR Allo	144	0.3773	0.2844	(----*---)
Allo	144	0.2765	0.3016	(----*---)
POOLED STDEV =		0.2715		0.30 0.40 0.50

(Auto = autograft; IR Allo = irradiated allograft; Allo = autograft)

Table IV.5b BETWEEN PROXIMAL AND DISTAL OSTEOTOMIES

	DF	SS	MS	F	p
Between groups	1	0.7225	0.7225	8.57	0.004
Within groups	412	34.7373	0.0843		
Total	413	35.4597			

OSTEOTOMY	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Proximal	198	0.3517	0.3045	(-------*-------)
Distal	216	0.4353	0.2768	(-------*-------)
POOLED STDEV =		0.2904		0.350 0.400 0.450

Table IV.5c BETWEEN AREAS ON THE SECTION (NEW BONE AROUND HOST OR GRAFT)

	DF	SS	MS	F	p
Between groups	1	1.8277	1.8277	22.39	0.000
Within groups	412	33.6321	0.0816		
Total	413	35.4597			

AREA	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
HN	207	0.4618	0.1907	(------*-----)
GN	207	0.3289	0.3562	(------*-----)
POOLED STDEV =		0.2857		0.300 0.360 0.420 0.480

(HN = new bone around host; GN = new bone around graft)

Table IV.5d DISTANCE FROM OSTEOTOMY SITE

	DF	SS	MS	F	p
Between groups	2	0.8232	0.4116	4.88	0.008
Within groups	411	34.6365	0.0843		
Total	413	35.4597			

SITE	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Close	138	0.4495	0.2806	(-------*-------)
Middle	138	0.3963	0.2902	(-------*-------)
Away	138	0.3402	0.2998	(-------*-------)
POOLED STDEV =		0.2903		0.300 0.360 0.420 0.480

(Close = samples close to osteotomy site; Middle = samples between "close and away"; Away = samples away from the osteotomy site)

Table IV.5e GENERAL LINEAR MODEL ANALYSIS OF DIFFERENCE BETWEEN GRAFT TYPES AND NEW BONE AROUND HOST AND GRAFT

SS	ADJ	ADJMS	F	P
2.186	2.09	1.05	17.48	0.00

Values are fractionated amount means and (standard deviations)

GRAFT	NEW BONE AROUND HOST	NEW BONE AROUND GRAFT
Autograft	0.52 (0.03)	0.59 (0.03)
Irradiated Allograft	0.46 (0.03)	0.29 (0.03)
Allograft	0.42 (0.03)	0.14 (0.03)

TABLE IV.6

ANALYSIS OF VARIANCE OF FRACTIONATED AMOUNT OF NEW BONE WITHIN THE HOST AND GRAFT.

Values are natural logarithm of:-

LABELLED / (LABELLED + UNLABELLED) counts.

Table IV.6a BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	8.985	4.492	7.75	0.001
Within groups	163	94.528	0.580		
Total	165	103.513			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	63	-2.5127	0.6641	(------*-------)
IR Allo	51	-2.0868	0.7353	(--------*-------)
Allo	52	-1.9929	0.8873	(-------*--------)
POOLED STDEV =		0.7615		-2.50 -2.25 -2.00

(Auto = autograft; IR Allo = irradiated allograft; Allo = allograft)

Table IV.6b BETWEEN PROXIMAL AND DISTAL OSTEOTOMIES

	DF	SS	MS	F	p
Within groups	1	0.241	0.241	0.38	0.537
Between groups	164	103.272	0.630		
Total	165	103.513			

OSTEOTOMY	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Proximal	79	-2.2590	0.7831	(----------*----------)
Distal	87	-2.1827	0.8029	(----------*----------)
POOLED STDEV =		0.7935		-2.40 -2.28 -2.16 -2.04

Table IV.6c BETWEEN AREAS ON THE SECTION (NEW BONE WITHIN GRAFT OR HOST)

	DF	SS	MS	F	p
Between groups	1	27.747	27.747	60.06	0.000
Within groups	164	75.766	0.462		
Total	165	103.513			

AREA	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
HO	130	-2.0039	0.6587	(--*--)
GO	36	-2.9959	0.7520	(----*-----)
POOLED STDEV =		0.6797		-3.20 -2.80 -2.40 -2.00

(HO = new bone within host; GO = new bone within graft)

Table IV.6d BETWEEN PERIOSTEAL AND ENDOSTEAL AREAS OF CORTICAL BONE

	DF	SS	MS	F	p
Between groups	1	0.096	0.096	0.15	0.697
Within groups	164	103.417	0.631		
Total	165	103.513			

	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
PERIOSTEAL	84	-2.1953	0.8011	(----------*----------)
ENDOSTEAL	82	-2.2433	0.7869	(----------*----------)
POOLED STDEV =		0.7941		-2.40 -2.28 -2.16 -2.04

Table IV.6e. DISTANCE FROM OSTEOTOMY SITE

	DF	SS	MS	F	p
Between groups	2	1.592	0.796	1.27	0.283
Within groups	163	101.921	0.625		
Total	165	103.513			

SITE	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Close	61	-2.3467	0.8361	(---------*---------)
Middle	56	-2.1317	0.7484	(---------*---------)
Away	49	-2.1599	0.7795	(---------*---------)
POOLED STDEV =		0.7907		-2.40 -2.20 -2.00

(Close = samples close to osteotomy site; Middle = samples between "close and away"; Away = samples away from the osteotomy site)

TABLE IV.7

ANALYSIS OF VARIANCE OF THE FRACTIONATED AMOUNT OF NEW BONE IN THE ENDOSTEAL REGIONS OF GRAFT AND HOST.

Values are:-

LABELLED / (LABELLED + UNLABELLED) COUNTS.

Table IV.7a BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	0.307	0.154	0.90	0.409
Within groups	275	47.110	0.171		
Total	277	47.417			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	90	0.4811	0.4066	(----------*----------)
IR Allo	105	0.4591	0.4065	(----------*----------)
Allo	83	0.3994	0.4307	(----------*----------)
POOLED STDEV =		0.4139		0.350 0.420 0.490 0.560

(Auto = autograft; IR Allo = irradiated allograft; Allo = allograft)

Table IV.7b BETWEEN PROXIMAL AND DISTAL OSTEOTOMIES

	DF	SS	MS	F	p
Between groups	1	0.368	0.368	2.16	0.143
Within groups	276	47.049	0.170		
Total	277	47.417			

OSTEOTOMY	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Proximal	133	0.4864	0.3947	(----------*----------)
Distal	145	0.4135	0.4288	(----------*----------)
POOLED STDEV =		0.4129		0.360 0.420 0.480 0.540

Table IV.7c BETWEEN AREAS ON THE SECTION (NEW BONE IN ENDOSTEAL AREA OF GRAFT OR HOST)

	DF	SS	MS	F	p
Between groups	1	13.443	13.443	109.21	0.000
Within groups	276	33.974	0.123		
Total	277	47.417			

BONE	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Host	158	0.6400	0.3345	(--*--)
Graft	120	0.1961	0.3713	(---*---)
POOLED STDEV =		0.3508		0.16 0.32 0.48 0.64

TABLE IV.8

ANALYSIS OF VARIANCE OF RATE OF NEW BONE FORMATION IN THE GRAFT IN THE TRANSVERSE SECTIONS

Values are the distances between the two labels.

BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	1746.58	873.29	269.91	0.000
Within groups	132	427.08	3.24		
Total	134	2173.66			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	45	7.630	3.116	(--*-)
IR Allo	45	0.000	0.000	(-*-)
Allo	45	0.000	0.000	(-*-)
POOLED STDEV =		1.799		0.0 2.5 5.0 7.5

(Auto = autograft; IR Allo =irradiated allograft; Allo = allograft)

TABLE IV.9

ANALYSIS OF VARIANCE OF FRACTIONATED AMOUNT OF NEW BONE IN THE GRAFT IN THE TRANSVERSE SECTIONS

Values are:-

LABELLED / (LABELLED + UNLABELLED) counts.

BETWEEN GRAFT TYPES

	DF	SS	MS	F	p
Between groups	2	2.4620	1.2310	17.91	0.000
Within groups	285	19.5849	0.0687		
Total	287	22.0469			

GRAFT	N	MEAN	STDEV	Individual 95% confidence intervals for means based on pooled STDEV
Auto	96	0.2552	0.3439	(-----*----)
IR Allo	96	0.0456	0.1672	(-----*----)
Allo	96	0.0762	0.2448	(-----*----)
POOLED STDEV =		0.2621		0.00 0.10 0.20 0.30

(Auto = autograft; IR Allo = irradiated allograft; Allo = Allograft)

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