ALLOGRAFT MICROVASCULAR EPiphySEAL PLATE TRANSPLANTS WITH SHORT-TERM IMMUNOSUPPRESSION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto

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Master of Science, 1999
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ABSTRACT

Current immunosuppressants are too toxic for long-term use in the context of allograft epiphyseal plate transplantation. Epiphyseal plates possess unique characteristics, however, that may permit long-term survival with only a short-term course of immunosuppression. A model for the free allograft microvascular transplantation of the rabbit proximal tibial epiphyseal plate was developed and validated. Transplants included the minimal amount of adjacent bone compatible with preservation of the epiphyseal plate vascular supply. This model was then used to assess the epiphyseal plate viability with a short-term immunosuppression regimen. Immunosuppression was provided by an initial 6-week course of continuous Cyclosporine therapy. Histologic and immunohistochemical evaluation of epiphyseal plate viability was performed four weeks after withdrawal. Epiphyseal plate grafts with bone contact were viable according to all outcome measures. Grafts without bone contact, however, were non-viable and showed evidence of advanced rejection. These results suggest that viability of allograft epiphyseal plates can be preserved following withdrawal of short-term immunosuppression provided the graft design and recipient site allow for adequate graft neovascularization.
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DEDICATION

This thesis is dedicated to Riina, my wife, whose support, patience, and encouragement was largely responsible for providing the motivation necessary to complete this project.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>DCAF</td>
<td>2, 4-bis(N,N'-di(carbomethyl)aminomethyl) fluorescein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon γ</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin 2</td>
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<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PTAH</td>
<td>phosphotungstic acid hematoxylin</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper lymphocytes</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>°Fix°CsA</td>
<td>epiphyseal plate transplants without bone contact and without short-term immunosuppression</td>
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Chapter One

1 INTRODUCTION
1.1 CLINICAL PROBLEM

Compromised function of an epiphyseal plate due to trauma, tumor, infection, or congenital malformation can result in significant musculoskeletal deformity. Correction of the subsequent clinical problems, such as limb length discrepancy and angular and rotational deformity is one of the greatest challenges faced by reconstructive surgeons. Various techniques are currently used to correct or minimize the extent of these deformities. These may include autogenous or allogeneic cancellous bone grafts, non-vascularized cortical allografts, vascularized bone and composite tissue transfers, and the Ilizarov technique of distraction osteogenesis. These solutions are not completely satisfactory for children since they do not adequately address the actively growing nature of the extremity.

Vascularized epiphyseal plate transplantation offers a potential advantage over the treatment modalities listed above. Replacement of the damaged or absent epiphyseal plate with a normal, functional one would permit continued growth of the extremity and more restore the natural development of the child's limb. Microvascular techniques have enabled the execution of experimental epiphyseal plate transplants with high levels of post-operative viability and subsequent growth.

Some clinical cases of autogenous epiphyseal plate transfers have now been reported, but the procedure is not common, largely because of the difficulty of obtaining suitable donor tissue. An attractive solution to this problem would be the use of cadaveric donor sites. This would eliminate donor site restrictions and allow for selection of the most appropriate epiphyseal plate for a given reconstructive problem.
A vascularized epiphyseal plate graft would consist of epiphyseal plate cartilage, bone, marrow elements and blood vessels, and possibly muscle, ligament and tendon. Survival of a vascularized, composite tissue transplant of this sort would require systemic immunosuppression in order to prevent rejection by the immune system of the recipient. The risks associated with current long-term immunosuppression regimens outweigh the potential benefits of non life-threatening procedures such as musculoskeletal reconstruction. Consequently, vascularized allograft transplantation of epiphyseal plates has never been described in humans.

Short-term use of immunosuppressants might prove ethically acceptable in the context of musculoskeletal reconstruction provided the viability and function of the transplanted epiphyseal plate could be assured after the withdrawal of immunosuppression. The unique characteristics of the epiphyseal plate render such a scenario at least theoretically possible. Proof of epiphyseal plate viability after withdrawal of short-term immunosuppression would be an important step towards the clinical application of vascularized allograft epiphyseal plate transplantation in humans.

1.2 ANATOMY AND PHYSIOLOGY OF THE EPIPHYSEAL PLATE

1.2.1 Overview

Epiphyseal plates are cartilaginous structures responsible for the longitudinal growth of immature bone. In the long bones, they are typically located between the epiphysis and the metaphysis in roughly the form of a disc oriented perpendicular to the longitudinal axis of the bone. Epiphyseal plates of this type are deemed primary epiphyseal plates, and will be the focus of remainder of this thesis. Secondary epiphyseal plates, or ossification centers, are spherical in
shape and responsible for endochondral ossification of the epiphyses. These will not be discussed further.

The longest bones of the axial skeleton have two epiphyseal plates, one at each of the proximal and distal ends. The contributions of the two epiphyseal plates to the total length of a given bone are not equal. In the human humerus, for example, the proximal and distal epiphyseal plates contribute 80 and 20 percent of the final length of the bone, respectively. The tibial epiphyseal plates are more evenly matched, with 55 percent of the total length provided by the proximal plate and 45 percent by the distal one\textsuperscript{177}. Smaller bones such as the phalanges have a single epiphyseal plate located at either the proximal or the distal end.

Epiphyseal plates effect skeletal growth by the process of endochondral ossification. This involves the two separate but tightly coupled processes of chondrogenesis by epiphyseal plate chondrocytes, followed by ossification. Ossified cartilage is remodeled into bone by neovascularization and invasion of bone cell precursors\textsuperscript{36}.

1.2.2 Histomorphology of the Epiphyseal Plate

The basic constituents of the epiphyseal plate include cells and intercellular matrix\textsuperscript{177}. Growth is a result of chondrocyte proliferation and production of matrix components. The matrix itself has several important roles in the function of the epiphyseal plate, including maintenance of its structure and as a medium for physiological processes.

Epiphyseal plate chondrocytes reside within irregular lacunae and undergo a series of well-defined maturation stages\textsuperscript{36} characterized by alterations in size, shape, proliferation rate and biosynthesis\textsuperscript{116}. The epiphyseal plate can be divided into zones, each characterized by different
microscopic and biochemical features. The three zones, resting, proliferative, and hypertrophic, each contain chondrocytes of a different maturational stage.\textsuperscript{116, 123}

The germinal (resting) zone of cells lies closest to the adjacent joint, and is in close proximity to the epiphyseal circulation under the epiphyseal bone plate (FIGURE 1; pg. 6). The epiphyseal capillaries, however, do not supply the germinal zone with oxygen but instead pass through it perpendicularly to terminate at the uppermost cells of the proliferative zone. Consequently, the oxygen tension of the germinal zone is relatively low. Cells of this zone are found singly or in pairs within abundant extracellular matrix, and are metabolically relatively inactive. The role of the germinal zone is unclear; it does not actively participate in longitudinal growth through cell proliferation, matrix synthesis, or calcification.\textsuperscript{117}

The proliferative zone is distinguished by longitudinal columns of flattened cells with low cytoplasmic volume, but a high nuclear-to-cytoplasm ratio. Longitudinal growth occurs only within this zone, and is a result of cell proliferation. The uppermost cell in each column serves as the progenitor cell, whose daughter cells also undergo reiterative divisions and contribute to total longitudinal growth. The proliferative zone has the highest oxygen tension of the epiphyseal plate. The matrix macromolecules secreted by proliferative zone cells include collagen type II and proteoglycans that contribute to a high coefficient of diffusion. Facile diffusion of oxygen and nutrients in this zone is likely important to support the high metabolic demands of proliferative zone chondrocytes. The matrix itself is applied between the cell columns, while successive cells in a column remain separated by a thin, transverse cartilaginous septum. All subsequent cellular and matrix changes facilitate the conversion of epiphyseal plate cartilage into bone.\textsuperscript{177}
FIGURE 1: Photomicrograph of unmanipulated epiphyseal plate

Legend:
A - Epiphyseal capillary
B - Epiphyseal bone plate
C - Germinative zone
D - Proliferative zone
E - Hypertrophic zone
F - Dying chondrocyte
G - Metaphyseal capillary

WHO stain, 250 X
As chondrocytes within a column progress distally, they gradually assume a more rounded shape, accumulate cytoplasm and begin synthesis of different matrix components. Chondrocytes with a low nuclear-to-cytoplasm ratio mark the beginning of the hypertrophic zone. The secretion of collagen type X into the lower portion of the hypertrophic zone has been implicated as an important step in the calcification of the matrix\(^{35}\). A concomitant event is the release of mitochondrial calcium into the extracellular matrix where it is taken up by extracellular matrix vesicles. These and other changes facilitate calcification of the matrix in the longitudinal intercellular septa of the lower hypertrophic zone. The transverse intercellular septa, however, do not calcify.

The final stage in the process of endochondral ossification occurs in the metaphysis. This region begins below the last transverse cartilaginous septum of each cell column of the hypertrophic zone. These lowermost septae are penetrated by terminal capillary loops from the metaphyseal vascular supply - a process associated with death of the hypertrophic chondrocytes. Osteoblasts secrete osteoid on the calcified longitudinal septae, forming the primary spongiosa. This initial cartilage/bone composite is subsequently remodeled and replaced by a mature secondary spongiosa, composed of lamellar bone, which no longer contains remnants of the cartilaginous precursor\(^{117}\).

1.2.3 Epiphyseal Plate Blood Supply

Although the epiphyseal plate is supplied with blood from several sources, researchers have sought to ascertain their relative importance with respect to epiphyseal plate function. Such knowledge has direct implications for epiphyseal plate transplantation as in a clinical scenario it may not always be technically practical to preserve all vascular sources (endosteal and periosteal) to the donor epiphyseal plate.
Descriptive studies of the epiphyseal and metaphyseal vascular supply of the epiphyseal plate were reported in the German literature during the mid-nineteenth century. These reports were concerned mostly with the effect of circulatory disruption on the histology of the epiphyseal plate. Technical limitations in the process of specimen preparation, however, led to speculative results.

The development of microangiographic techniques in the mid-1950's facilitated detailed study of the blood supply of bone. Using these methods, the three sources of arterial supply to the epiphyseal plate were evaluated: the epiphyseal periosteal, metaphyseal periosteal and endoteal supply from the nutrient artery. The epiphyseal arteries were shown to enter the secondary center of ossification, arborize within the epiphysis, and terminate at the summit of columns of the proliferative zone after piercing the subchondral bone layer. The basic arrangement of these terminal branches was described as, "a vascular ceiling under the roof of the bone plate". The nutrient artery enters the diaphysis, usually close to its midpoint, and terminates in vascular capillary loops below the last transverse cartilaginous septa of the hypertrophic zone. These capillaries arise from vertically-oriented arterioles, and supply mainly the central portion of the epiphyseal plate. The circumferential metaphyseal arteries, which are derived from periosteal arteries, supply the peripheral portion of the hypertrophic zone.

Selective disruption of the epiphyseal or metaphyseal arterial systems allowed differentiation of their respective roles in the control of osteogenesis. Obliteration of the epiphyseal vascular supply caused eventual destruction of the plate and cessation of longitudinal bone growth. Disruption of the metaphyseal vascular supply, however, resulted in very different findings. The germinal and proliferative zones were unaffected by the change; all processes of
cartilage formation, palisading and calcification continued in a relatively normal fashion. After several days, the hypertrophic zone became thickened with marked widening of the region of calcified cartilage. This change to the hypertrophic zone was reversible if vascular supply was re-established to the metaphyseal side by vascular ingrowth. These findings lead to the conclusion that the metaphyseal circulation had little role in the promotion of epiphyseal plate growth, but was important for ossification and remodeling and of the cartilaginous columns. Recent studies using scanning electron microscopy have confirmed that the metaphyseal vessels are not necessary for epiphyseal plate growth and survival, but regulate the size of the epiphyseal hypertrophic zone and direct metaphyseal osteoprogenitor cells to the scaffolds of the calcified cartilage matrix.115

Detailed understanding of the vascular supply to the epiphyseal plate provided the groundwork necessary for experimentation with microvascular transplantation of epiphyseal plates29, 30. The restoration of vascular supply to both the epiphyseal and metaphyseal sides of the epiphyseal plate is essential for normal function after transplantation. However, in the absence of the nutrient artery revascularization, growth continues after transplantation due to vascular ingrowth that is able to reconstitute the metaphyseal circulation.199

1.3 PHYSIOLOGY OF BONE GRAFTS

Although transplantation of epiphyseal plates is the focus of this study, the process shares many characteristics with the grafting of bone. The bony component of the epiphyseal plate transplant is essential for provision of the vascular network to the epiphyseal plate and for the structural integrity of the graft itself. In a clinical scenario, solid union of graft bone with the recipient site would be necessary to effect stability of the entire construct and thereby permit the epiphyseal plate to contribute to longitudinal growth. Because vascularized epiphyseal plate
transplants share many characteristics and considerations with vascularized bone grafts, a discussion of the biological processes important to the technique of bone grafting will be presented.

1.3.1 Healing of bone grafts

Non-vascularized bone grafts, so called "conventional" bone grafts, are routinely used in surgical practice. With this technique, the bone tissue is transferred without preservation of the vascular supply. With conventional bone grafts, only a small percentage of osteocytes survive transplantation, but the bony components that remain serve as structural supports during the healing process. In the initial post-operative period, the graft serves as a scaffold for bony ingrowth of the host tissues. The graft stimulates bone formation through the process of osteoconduction, the mechanisms of which are poorly understood. Osteoconductive recruitment of host blood vessels into the graft results in bone formation. Ingrowth of new blood vessels and resorption of the nonviable bone must occur before replacement of the graft tissue with viable cells and new matrix. This process is efficient in cancellous bone grafts because they provide an open structure that permits the ingrowth of new vessels and facilitates diffusion of nutrient substances, thus enhancing osteocyte and osteoblast survival. Nonvascularized cortical grafts are dense, and initially undergo resorption that predisposes them to fatigue fractures before significant new bone formation occurs.

Patterns of incorporation and regeneration differ somewhat in cortical and cancellous bone grafts. With cancellous bone grafts, these processes can be divided into three general phases: *Phase 1 (weeks 0-3) - This phase is characterized by vascular ingrowth and progenitor mesenchymal cell invasion. Revascularization occurs almost entirely via newly developed
vessels of host (recipient site) origin. The rate of this process in rabbits has been investigated using intravital microscopic techniques. Vascular penetration into cancellous bone grafts occurred at rates up to 0.4 mm/day. Ingrowth into cortical bone was slower, with a maximum rate of 0.15 to 0.3 mm/day.

*Phase 2 (weeks 3-12)* – The newly-arrived mesenchymal cells differentiate into osteogenic cells. Osteoblasts line the edges of dead trabeculae and surround them with osteoid. The entrapped cores of necrotic bone are subsequently resorbed by osteoclasts. Through this combination of osteoblastic appositional new bone formation and osteoclastic resorption of the graft trabeculae, necrotic donor bone is eventually completely replaced with new, viable bone.

*Phase 3 (months 3-6)* – Through the activity of osteoclasts and osteoblasts, trabeculae are remodeled and reoriented into a mature pattern. As in normal, unmanipulated bone, this process is influenced by the mechanical and loading forces to which the graft is subject. Significant remodeling is found to occur only after vessel density in the graft reaches a level approximately equal to that before grafting.

The incorporation of cortical bone grafts differs from that of cancellous bone grafts in several major ways. Vascular ingrowth into the cortical grafts is much slower. The porous architecture of cancellous bone readily permits the ingrowth of vessels from the host bed. The higher density of cortical bone, however, acts as a barrier to diffusion and vascular ingrowth. In addition, cortical grafts differ in the mechanism of repair; osteoclastic resorption precedes osteoblastic new bone formation. Osteoclasts form cutting cones within the donor Haversian canals, thereby enlarging them, and new bone is formed behind - the process of creeping substitution. Finally, cortical grafts do not undergo complete substitution with viable bone; they
become admixtures of necrotic graft bone and viable bone. The non-viable donor bone becomes essentially sealed within the architecture of the new, vascularized bone.

The epiphyseal plate graft used in this particular study contains both cortical and cancellous bone, as does the iliac crest recipient site. Thus, on a basic level, elements of both cortical and cancellous incorporation may well be anticipated. However, the blood supply to the epiphyseal plate graft will be restored by microvascular anastomoses. Consequently, the process of bony incorporation in this study will have more in common with that of vascularized bone grafts than that of the non-vascularized grafts outlined above.

1.3.2 Vascularized Bone Grafts

In contrast to non-vascularized grafts, experimental and clinical studies indicate that immediate vascularization of cortical autografts improves osteocyte survival and enhances bony incorporation\(^{20, 162, 174, 187}\). Healing of the graft to recipient bone is facilitated without the usual slow process of resorption and regeneration from host cells (creeping substitution)\(^{90}\). This feature results in clinically more rapid stabilization of bony segments and less loss of structural rigidity.

Although the vascularized bone grafts have their own blood supply, vascular ingrowth from the recipient site still occurs, in the same manner that vascular continuity is re-established across a rigidly fixed fracture site in a long bone. In this process, cutting cones extend across the osteotomy site for direct osteon to osteon hookup. Trailing osteoblasts, accompanied by a vascular network, lay down new bone. Gaps between the graft and recipient site are filled quickly with osteoid. Lamellar bone is formed and then bridged by secondary osteons\(^{161}\). Remodeling may be seen at approximately four weeks post-transplantation by proliferation of Haversian canals along the longitudinal axis of the bone.
In situations where rigid fixation of a graft (or fracture) is not achieved, the reparative process occurs through an intermediate stage involving callus formation. This typically exhibits formation of new bone on a cartilaginous template, as in the external callus, and membranous bone formation, in which bone is formed directly from mesenchymal cells as in the periosteal callus\textsuperscript{161}. In the current experiment, the graft is not secured with true rigid fixation. Consequently, bone healing by callus formation may be seen to a greater degree than that of primary bone healing.

1.4 HISTORY OF EPIPHYSEAL PLATE TRANSPLANTATION

1.4.1 Transplantation of Non-Revascularised Epiphyseal Plates

Attempts to transplant epiphyseal plate cartilage early as the turn of the century. Prior to the development of microvascular techniques, transplantation of epiphyseal plates was performed without surgical revascularization. Survival of these grafts depended on their ability to acquire nutrients by diffusion from the recipient bed until adequate neovascularization occurred.

The first published epiphyseal plate transplant described the use of the canine distal ulnar epiphysis\textsuperscript{106}. Further experimental non-vascularized autogenous and allogeneic epiphyseal plate transplants were reported in both the English and German literature in a variety of models\textsuperscript{21, 68, 99, 100, 156, 197, 202, 228, 230}. Success, in terms of continued longitudinal growth of the transplanted tissue, was sporadic and results were unpredictable. Both continued longitudinal growth\textsuperscript{78, 105, 232} and diminished or absent growth\textsuperscript{178, 198, 234} were reported. Conflicting results in these studies were likely due to the variety of graft designs in different animal models. Fundamentally, however, the lack of significant longitudinal growth was certainly related to ischemia of the grafts resulting from transplantation without a supporting vascular supply. The
best results were achieved when only a small amount of epiphyseal cartilage was transplanted with a minimal layer of attached bone, thereby permitting rapid revascularization on both sides of the epiphyseal plate. Even so, the generally unsatisfactory results of non-vascularized epiphyseal plate transplants precluded their development into a clinically viable surgical modality.

1.4.2 Transplantation of Revascularised Epiphyseal Plates

The development of reconstructive microvascular surgery over the past three decades has made it possible to transfer large blocks of tissue isolated on a single vascular pedicle. These free tissue transfers can be performed with high levels of post-operative viability and are now an established part of clinical practice. The blood supply to the epiphyseal plate has been understood for many years. Once techniques were developed to dissect epiphyseal plates with their blood supply intact, transplantation with immediate revascularization became feasible. Preliminary support for the success of these procedures was provided by studies documenting post-operative growth of the epiphyseal plates in replanted digits of children.

Experimental studies in animal models, however, enabled evaluation of epiphyseal plate viability and function in response to controlled interventions. Most of this work was performed using autograft orthotopic or heterotopic transplants.

1.4.2.1 Experimental autograft vascularized epiphyseal plate transplants

1.4.2.1.1 Canine distal ulna model.

The first experimental vascularized epiphyseal plate transplants were performed using canine models. Vascularized and non-vascularized transfers of the distal ulna with its epiphysis were used to establish the role of the endosteal and periosteal metaphyseal blood supply. The revascularized orthotopic transfers showed impaired growth, achieving only 63% of the total
increase in length seen in the non-operated control ulnas. This relatively impaired growth, however, was significantly greater than that seen in the non-vascularized ulnar grafts. The authors thought that only the metaphyseal blood supply was reestablished in their model and thus assumed that this contributed to the growth potential of long bones. In fact, later studies have demonstrated that the epiphyseal blood supply is also reestablished, although only poorly, in this model.\textsuperscript{217, 219, 220}

A subsequent study was undertaken to evaluate growth potential with exclusion of the nutrient artery (preservation of the periosteal blood supply only)\textsuperscript{61}. The extent of growth (69\% of controls) was comparable to that seen in the previous study in which the nutrient artery was preserved. These results suggested that the inclusion of the nutrient artery in vascularized epiphyseal transplantation did not offer advantages in terms of the growth potential beyond that obtained with the periosteal circulation alone.

The canine distal ulna was used as the basis for a subsequent series of investigations of vascularized epiphyseal plate transfers\textsuperscript{27, 28} and included detailed macroscopic assessments of vascularity. The graft incorporated the distal 4 cm of the ulna including the physis which was isolated on the median artery and a dorsal vein. With this model, the nutrient artery to the ulna was excluded and the blood supply to the graft was through the periosteal and perichondrial vessels. The vascular studies performed by Bowen and coworkers showed that the blood supply to the epiphysis was maintained in this model, albeit by small, terminal vessels. Attempts were made to further define the role of the epiphyseal and metaphyseal vascular supply by defining several experimental sub-groups. These included transfers with periosteal stripping of either the epiphysis, the metaphysis, or not at all. Grafts with stripping of the epiphysis, metaphysis, or without stripping demonstrated growth of 42\%, 76\%, and 85\% of the control ulna, respectively.
In the free transfers, frequent complications such as skeletal collapse, deformity, and growth failure prevented quantitative comparisons of growth. It was apparent, however, that continued growth and structural integrity of the transfers were maintained only with preservation of the blood supply to both the epiphysis and metaphysis.

The second investigation in this series evaluated the use of the canine distal ulna epiphyseal plate as either a replacement for, or an accessory to, the existing ipsilateral radial epiphyseal plate. Vascularized grafts of the distal ulna were used to reconstruct defects of either the ipsilateral distal radius (replacement epiphyseal plate) or the radial diaphysis (accessory epiphyseal plate). Although epiphyseal plates transferred to the radial diaphysis did contribute in an adjunctive manner to the total growth of the radius, complications in these animals generally interrupted the experiment at approximately 8 weeks post-transplant. Fractures and malalignment occurred at the accessory epiphyseal plate, despite relief from weightbearing with the use of splints. The authors attributed these developments to the vulnerable location of the accessory plate where it was subjected to increasing moments of force as a result of longitudinal growth. These results highlighted the complicated biomechanical issues inherent to the transplantation of epiphyseal plates, especially with transfers to a heterotopic location.

1.4.2.1.2 Canine proximal fibula model.

The canine proximal fibula epiphyseal transfer was developed to simulate the clinical use of the human proximal fibula as a vascularized bone transfer with growth potential. The anatomical basis for the isolation of the proximal fibula, including the epiphysis, on the popliteal vessels was established with the use of angiography, plastic injections and radioactive microsphere injections.
Orthotopic transfers with revascularization grew at near-normal levels\textsuperscript{38, 150}. As seen in previous studies, grafts without revascularization suffered rapid loss of viability and did not grow. Encouraged by these results, Nettleblad and coworkers\textsuperscript{149} used the distal radius as a distant, heterotopic recipient site to simulate a clinically relevant reconstruction. Both a short-term study (6 week followup)\textsuperscript{152} and a long-term study (6 month followup)\textsuperscript{149} were performed. In both investigations, the amount of growth was variable (10\% to 80\% of the control fibula) and complications, including fractures and destruction of the epiphyseal plate, were observed after removal of the casts at 5 weeks. The size discrepancy between the graft and the radius, as well as effects of altered loading, were implicated in the impaired growth of the transplants.

1.4.2.2 Clinical autograft vascularized epiphyseal plate transplants

Elective pediatric microvascular transplants, such as toe-to-hand and whole-joint transfers have demonstrated continued epiphyseal plate growth. In a report on the results of three toe-to-hand transplants, growth was documented at 89\% of normal in the phalanges\textsuperscript{155}. Near normal rates of growth were reported in some series of whole-joint transplants\textsuperscript{136, 191} although premature epiphyseal plate closure was a prominent feature in some cases\textsuperscript{157}.

The microvascular transplantation of isolated epiphyseal plates has not been a commonly performed clinical procedure. Published reports of these procedures have mainly described the use of the proximal fibula with its epiphysis isolated on the peroneal or anterior tibial vessels\textsuperscript{52, 170, 221}. Although successful results have been reported following upper extremity reconstruction\textsuperscript{119}, the amount of growth was often much less than in normal controls\textsuperscript{170}, was unpredictable, and associated with premature closure of the epiphyseal plate\textsuperscript{221}. 
Although small series' have documented the use of other donor sites with growth potential, including the vascularized apophyses of the scapula and the iliac crest, the only expendable epiphyseal plate of reasonable size in humans is that of the proximal fibula.

1.4.2.3 Allograft vascularized epiphyseal plate transplants

In a clinical setting, autograft vascularized epiphyseal plate transplants only have therapeutic relevance when performed using a heterotopic donor site. Potential donor sites for autogenous epiphyseal plates are limited, however, and include only the phalanges and the proximal fibula. Consequently, interest has developed in allograft transplantation of epiphyseal plates. The use of cadaveric tissue would allow replacement of a specific epiphyseal plate with a graft from an orthotopic location in the donor. Such a scenario would minimize donor-site morbidity in the recipient and provide grafts from a similar biomechanical and physiological environment to the epiphyseal plate undergoing replacement. In addition, allografts may permit creative use of donor tissue from donors of different ages, sex, or growth potential.

The behavior of allograft epiphyseal plates following microvascular transplantation into immunosuppressed hosts has been investigated in several studies. Boyer and coworkers used a modification of the rabbit knee transplant model. The rabbit knee, consisting of the distal femur and proximal tibia, each with their epiphyses, was isolated on the popliteal arteriovenous pedicle. Knees were transplanted between rabbits determined to be allogeneic by molecular haplotyping. In animals immunosuppressed with Cyclosporine A, growth of the vascularized allografts was not significantly different from the vascularized autografts. This finding was corroborated by the microscopic analysis, which showed histologically viable epiphyseal plates.
Two additional studies\cite{19,20} also used a modification of the rabbit knee model to investigate the behavior of epiphyseal plates following allograft transplantation. The transplanted epiphyseal plates were morphologically normal upon histological examination but grew significantly less than those in the normal, control limbs. Although the extent of growth varied in the allograft studies, a consistent finding was that the potential for growth (and preservation of viability) of a vascularized allograft epiphyseal plate transfer was maintained provided the immunosuppression was adequate.

1.5 MEASUREMENT OF EPiphySEAL PLATE VIABILITY

Studies of epiphyseal plate transplantation have made relatively little use of direct quantitative indices of epiphyseal plate viability. Most have relied on epiphyseal plate growth as an outcome, coupled with histological assessments.

Fluorochrome labels given to a growing animal become deposited in the newly mineralized bone in the metaphyseal area immediately below the epiphyseal plate\cite{160,173,176}. With further growth, these labels are forced distally, further into the metaphyseal area away from the epiphyseal plate. Thus, visualization of the different labels gives an indication of graft viability at specific points in time – a technique used in many previous studies of vascularized bone allografts\cite{33,94,109,168,185}. This technique is not foolproof, however. Necrotic bone has been shown to incorporate and retain tetracycline labels\cite{214}. The labels are also sensitive to the histological processing regimen, and may be removed by certain fixatives and decalcifying agents\cite{213,224}.

We anticipated difficulty relying on growth alone as an indicator of viability in the current study, especially following withdrawal of immunosuppression. Measured growth provides an
indication of epiphyseal plate viability over a time interval; it does not give information about viability at a specific point in time, especially if the results of the fluorochromes are imperfect. Growth of small magnitude is difficult to discern with macroscopic measurement techniques, especially if variances are high. In addition, we anticipated that the graft in the current model might experience limited growth due to bracketing of the epiphyseal plate within a recipient site of relatively fixed dimensions. Consequently, although epiphyseal plate growth was quantified in the current study, it was used as an adjunctive rather than as a definitive indicator of viability.

Descriptive histology does provide information regarding the viability of epiphyseal plates, but the interpretation is subjective and difficult to quantify. With histological tissue sections, a pristine epiphyseal plate, or one that is frankly necrotic, can be easily identified. The viability of epiphyseal plates with disordered architecture or questionable histological features is less clear.

Heights of individual zones of the epiphyseal plate have been shown to change in response to various insults. These include ischemia, low-grade immunological rejection, and altered loading. Although quantitative, measurement of epiphyseal plate heights provides non-specific information that is not necessarily reflective of viability.

Various cell-cycle specific markers have been used in the assessment of epiphyseal plate behavior. These include incorporation of tritiated thymidine, 5-bromo-2'-deoxyuridine (BrdU), Ki-67, and PCNA. Histological processing of skeletal tissues poses special technical challenges due to the need for decalcification prior to embedding and sectioning of the tissue. The decalcification step can reduce the immunoreactivity of cellular antigens, rendering immunohistochemical detection difficult or impossible. One particular marker, 5-bromo-2'-deoxyuridine (BrdU) has been
shown to retain immunoreactivity following the type of tissue fixation and decalcification required for our histological specimens.  

Bromodeoxyuridine is a thymidine analogue specifically incorporated into the nuclei of cells in the S-phase (DNA synthesis) of the cell cycle. Presuming all cells in this phase will proceed to mitosis, an indication of the relative number of proliferating cells can be obtained. This technique has proven valuable to study growth kinetics of normal and malignant tissues. A quantitative, labeling index can easily be calculated by expression of the number of labeled proliferative zone nuclei as a fraction of the total number of proliferative zone nuclei. This method has several advantages over other indicators of epiphyseal plate viability in that it is quantitative, objective, and specific for proliferating (viable) chondrocytes. Antibody detection kits for BrdU are commercially available, and are suitable for use with decalcified paraffin-embedded tissue.

1.6 TRANSPLANT IMMUNOLOGY

1.6.1 Acute rejection

The rejection of foreign tissue by an organism is a highly coordinated, multi-step process mediated by the immune system of the recipient. Components of the cellular immune system, including T-lymphocytes, B-lymphocytes, and accessory cells are of major importance in the processes leading to the eventual destruction of the allograft. Initiation of the rejection response depends on the ability of the recipient’s immune system to discriminate ‘self’ tissues from ‘non-self’. Key to this process are the glycoprotein antigens of the Major Histocompatibility Complex (MHC) expressed on cell surfaces. The role of the MHC is the presentation of antigens in a form that is accessible to the T-cell receptor (TCR) of circulating T-lymphocytes.
Two distinct groups of MHC antigens, known as Class I and Class II, have been described. Class I antigens are expressed on the surface of all nucleated cells, and contain a heavy chain that exhibits a high degree of polymorphism within individuals of the same species. These antigens are the target antigens recognized by CD8 cytotoxic T-lymphocytes \(^{14}\) (CTL) during acute graft rejection.

Class II antigens, in contrast, are expressed constitutively on only certain cell types. These include B-lymphocytes and certain accessory cells (e.g. dendritic cells) and can be induced on other cell types such as macrophages, epithelium and vascular endothelium by interferons, interleukins, and leukotrienes\(^ {172}\). Class II antigens are recognized by T-lymphocytes bearing the CD4 surface protein, alternatively known as T-helper cells\(^ {165}\).

It is through interactions of the T-cell receptor (TCR) on host T-lymphocytes with MHC antigens of donor origin that the activation process begins\(^ {23}\). Recognition of alloantigen, however, is insufficient to induce a full immune response. According to the two-signal model of alloreactivity, T-cell immunity, and therefore rejection, is triggered by two signals - alloantigen, and a signal (co-stimulation) from specialized antigen presenting cells\(^ {128}\). MHC Class I antigens are present on most donor cells within the transplant, including osteoblasts, osteocytes, hematopoietic tissue and vascular endothelium. These serve as an abundant source of alloantigen for the sensitization of, and subsequently as targets of, cytotoxic T-lymphocytes. Certain consequences of the transplantation process, such as ischemia and increased production of cytokines, lead to increased expression of Class I antigens.

Activation of T-helper cells occurs by interaction with donor leukocytes bearing foreign Class II antigens, and/or by recognition of alloantigens presented by recipient APCs in the context of self MHC\(^ {200}\). Vascular endothelium, the integrity of which is vital to allograft
perfusion, prominently displays MHC Class II, and may also contribute to the immunogenic potential of vascularized allografts\textsuperscript{130}. Expression of Class II antigens is important in the development of the immune response; activated T-helper cells initially mediate the response, are required for activation of CTLs, and help B cells produce cytotoxic antibody.

Interaction of the TCR with MHC-bound alloantigen leads to expression of IL-2 receptors on the T-cell surface. Activated APC's send a signal, in the form of IL-1, which stimulates release of IL-2 by the lymphocytes. This product activates the IL-2 receptors in both an autocrine and paracrine fashion. Binding leads to clonal expansion of both Th and CTL and differentiation to mature cell forms. A complicated cascade of lymphokines and cytokines augments the response and recruits participants into the allograft.

The subsequent inflammatory response established in the graft is characterized by large numbers of invading lymphocytes\textsuperscript{42}. Gamma-interferon recruits leukocytes to the graft, and augments MHC Class I and II antigen expression. This enhanced expression makes the transplant more vulnerable to targeting by activated T lymphocytes. Several co-existant processes contribute to destruction and death of the allograft. Complement and the coagulation system induce and propagate inflammation, vascular leakage, and microthrombus formation. Lysis of target cells is effected by toxic proteins, such as perforins and proteolytic enzymes, released by activated CTLs\textsuperscript{222}. Lymphokines may also regulate non-immune cells, resulting in osteoclast activation, bone resorption, and biologic failure of the graft\textsuperscript{110}. 
1.7 IMMUNOLOGICAL CHARACTERISTICS OF BONE AND CARTILAGE ALLOGRAFTS

Vascularized epiphyseal plate transplants are by necessity composite grafts, consisting of epiphyseal plate cartilage, bone and periosteum, marrow elements, blood vessels, and small portions of muscle, ligament and tendon attachments. The various component tissues of these grafts differ in their antigenicity, and also the rapidity with which they suffer immunological compromise following transplantation\(^{46, 93, 138}\). This differential susceptibility to destruction by the host immune system is an important feature that is exploited in the design of the model used in this study.

1.7.1 Antigenicity of vascular endothelium

The survival of vascularized epiphyseal plate (and bone) transfers, like renal and cardiac transplants, depends on the integrity of the vascular pedicle and microcirculation within the graft. Immunologic or other insults to these structures pose great risk to the viability and function of the transplanted organ. Investigators have demonstrated that endothelium is a central target for attack by the recipient immune system\(^{113, 206}\), and is one of the first components of the graft to suffer damage following transplantation in the absence of adequate immunosuppression.

The central role of vascular endothelium in allograft rejection is not surprising. Donor vascular endothelium is the first point of host immune sensitization by virtue of cell surface alloantigens. Endothelial cells have been shown capable of direct T-cell activation by MHC Class I\(^2\). A potentially more important pathway, however, involves alloactivation of Th cells by endothelial MHC II. Expression of these antigens is induced on endothelial cells concomitantly with the generation of inflammation by mediators such as IFN-\(\gamma\).
Functional evidence of the immunological role of endothelium was provided in a study of arterial allografts. Grafts stripped of endothelium exhibited long-term patency in the absence of immunosuppression, whereas those with intact endothelium suffered occlusion within 5 days of transplantation. The immunologic behavior of vascular endothelium has direct implications for vascularized epiphyseal plate transplantation. Rejection of the microcirculation and/or vascular pedicle would result in graft ischemia and necrosis even if bone, cartilage and other supporting cells were unaffected by rejection themselves. This scenario has been suggested by findings that osteocyte death in vascularized bone allografts paralleled areas in which the microcirculation had been lost.

Vascular endothelium does not undergo replacement with like cells of donor origin. Studies of human renal allografts demonstrated only partial endothelial replacement in a small percentage of cases. Long followup periods greater than 26 years post-transplant revealed the bulk of the vascular and renal tubular epithelium retained the donor phenotype. In most cases, withdrawal of immunosuppression renders the organ susceptible to immunological attack. If short-term immunosuppression is to be successful, the graft must acquire a new blood supply of host origin. Although this process does not occur following transplantation of parenchymal organs such as kidneys, the unique ability of grafted bone to undergo neovascularization and remodeling may permit such an event to take place.

1.7.2 Antigenicity of Bone grafts

Vascularized allografts of bone and cartilage resemble more conventional parenchymal allografts in that they are immediately perfused with immunocompetent recipient cells. This characteristic allows for immediate host sensitization by endothelial cells within the graft.
vasculature, and by exposed and/or circulating hematopoietic donor cells. With non-vascularized
grafts, the recipient is exposed to donor antigens during processes of post-operative inflammation
and wound repair. This process is much slower\textsuperscript{208}, and likely requires processing of donor
antigens by recipient APCs in order for activation.

As in allografts of other solid organs, the antigenicity of bone is primarily due to surface
HLA antigens present on the heterogeneous population of cells within the graft. These may
include cells of osteogenic, chondrogenic, fibrous, vascular, and hematopoietic origin\textsuperscript{161}.
Studies suggest that marrow cells and passenger leukocytes may be the most immunogenic
component\textsuperscript{65, 146}. Marrow and dendritic cells are known to express MHC Class II antigen\textsuperscript{56, 103},
and can induce potent allograft reactivity\textsuperscript{71}.

Class I, Class II, minor histocompatibility, and non-major histocompatibility complex
antigens have been identified on osteocytes\textsuperscript{147, 194}. Indeed, these are among the first cells to
necrose after allograft transplantation, but this effect may be due to ischemia following rejection
of the local microcirculation, rather than primary destruction of the osteocytes themselves\textsuperscript{94}.
Matrix macromolecules, such as proteoglycan subunits, link proteins, and collagen may be
additional (weakly) antigenic components\textsuperscript{69}.

Evidence of both humoral and cellular immune responses has been demonstrated following
allograft bone transplantation\textsuperscript{79, 118, 146}. Humoral responses were directed against MHC
products following allograft transplantation of fresh mouse bone\textsuperscript{102} or freeze-dried human
bone\textsuperscript{80}. Recipient immune responses following vascularized transplantation were both faster and
more intense than in non-vascularized transplants\textsuperscript{118, 208}. Strong cellular and humoral immune
responses were seen beginning five days post-transplant, which peaked in intensity at day fourteen\textsuperscript{118}.

From a functional standpoint, nonvascularized bone allografts have structural properties similar to those of autografts; the allograft acts as scaffold for bony ingrowth of the host tissues. Stimulation of host bone by osteoconduction is much reduced and variable. For allograft cortical bone, the process is generally the same as for autografts although creeping substitution occurs much more slowly\textsuperscript{161}. The factors that stimulate host blood vessels to proliferate, invade the graft, and develop into cutting cones appear to be diminished, absent or severely suppressed\textsuperscript{89}. All subsequent processes appear delayed and limited in degree. Even if the allograft is successfully incorporated, the proportion of necrotic graft bone to viable host bone is greater in allogeneic grafts.

1.7.3 Antigenicity of Epiphyseal Plates

In contrast to most other tissue types, long-term survival of allograft cartilage has been demonstrated in the absence of immunosuppression\textsuperscript{66, 204}. Hypotheses were formulated to account for the seemingly “immunologically privileged” nature of cartilage. The basic theories forwarded were:

1. Components of cartilage are not immunogenic\textsuperscript{86}.

2. Certain components of cartilage may be immunogenic but they are protected from the activity of the host immune system\textsuperscript{15}.

Several investigations have shown that components of cartilage, particularly chondrocytes, possess MHC cell surface antigens that render them immunogenic. Chondrocytes in sheep were shown to possess the same histocompatibility antigens found on peripheral blood lymphocytes\textsuperscript{67}.
In rats and rabbits, chondrocytes isolated from matrix stimulated immune responses because of MHC-associated antigens, and stimulated allogeneic lymphocytes in vitro. These antigens have been classified as MHC Class II. In a study of rabbit articular chondrocytes in vitro, cells exhibited efficient antigen-presenting function mediated by Class II histocompatibility antigen and were able to stimulate allogeneic lymphocytes.

Given that chondrocytes possess antigens that render them immunogenic, and are capable of antigen-presentation and T-lymphocyte activation, the intercellular matrix is the likely source of the unique immunologic characteristics of cartilage. The matrix of cartilage is dense; the theoretical pore size is so small as to limit the egress of large antigens and decreases the rate of ingress of antibodies or cells. This suggests that as long as the matrix remains intact the tissue is immunologically privileged.

The capacity of the intercellular matrix to serve as a protective barrier has been validated by experimental studies. Following allograft transplantation, small grafts of intact cartilage remained viable, whereas chondrocytes enzymatically freed of matrix were surrounded by inflammatory infiltrates and resorbed.

1.7.4 Histologic Features of Rejection in Vascularized Grafts of Bone and Cartilage

Allograft vascularized knee joints survived an average of 12 days following allograft transplantation in non-immunosuppressed rabbits. At twelve days post-transplantation, clear features of acute vascular rejection were found in the pedicle while evidence of cellular rejection in bone or cartilage was absent. The microcirculation, however, suffered compromise prior to occlusion of the vascular pedicle. Serial bone scans showed progressive decline and finally absence of radionucleide activity within graft, although arteriography or histology demonstrated
the pedicle still to be patent, albeit narrowed and with damaged endothelium. These findings were consistent with the theory that survival is limited not by rejection of the graft itself, but by rejection of the transplanted vasculature.

The rat knee transplant model provided clearer delineation of the histological changes associated with the rejection of vascularized bone and cartilage allografts. The use of inbred rat strains permitted the transplantation of bone grafts across both strong and weak histocompatibility barriers.

In the absence of immunosuppression, rat knees transplanted across a strong histocompatibility barrier demonstrated significant histological changes five days after transplantation. Changes to the epiphyseal plate included eosinophilia of chondrocytes and decreased staining of matrix proteoglycans. The actual structure of the epiphyseal plate, however, remained essentially unchanged. In the marrow, loss of the normal structural pattern was accompanied by capillary disruption and massive extravasation of red blood cells. Osteoblasts in the primary spongiosa were absent, replaced instead by cellular debris and fibrin deposition. These changes were accentuated 7 days post-transplantation. At this time, osteocytes and chondrocytes were either necrotic or absent, microcirculatory flow had ceased, and the pre-sacrifice fluorochrome label was not visible.

Transplantation across a weak histocompatibility barrier resulted in a less intense immunological response but allowed more precise tracking of the rejection process. Osteoblasts, marrow cells, and the microcirculation were the first components affected. Osteocytes remained viable as long as the local microcirculation was intact. Massive thickening was noted in the hypertrophic zone of the epiphyseal plate, although the chondrocytes themselves remained viable. Similar findings have been reported following disruption of the metaphyseal
circulation\textsuperscript{26}. Epiphyseal plate thickening was not noted when transplants were performed across strong histocompatibility barriers\textsuperscript{94,112}, because rejection of the allograft was so vigorous that it rapidly resulted in total necrosis due to vascular damage.

Based on these findings, a semi-quantitative "rejection index" was devised\textsuperscript{167}. Each of the five rejection parameters (Table 1, pg. 30) was graded from 1 to 5, where the highest score coincided with the absence of rejection. The total score, out of 25, was found to have strong correlation to the extent of rejection in the specimens. Although this index was not designed for direct evaluation of epiphyseal plate viability, it will be included in the current study as an adjunctive test.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{REJECTION CRITERIA} & \textbf{SCORE} \\
\hline
Red cell extravasation & 1 - 5 \\
Marrow cell population & 1 - 5 \\
Fibrin deposition in spongiosa & 1 - 5 \\
Staining of cartilage proteoglycans & 1 - 5 \\
Pre-sacrifice fluorochrome label & 1 - 5 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{REJECTION SCALE} & \\
\hline
Score & Interpretation \\
\hline
23 – 25 & No rejection \\
18 – 22 & Mild rejection \\
11 – 17 & Moderate rejection \\
5 - 10 & Intense rejection \\
\hline
\end{tabular}
\end{table}

1.8 STRATEGIES TO PREVENT REJECTION OF ALLOGRAFT VASCULARIZED TRANSPLANTS OF BONE AND CARTILAGE

The general strategies that might be used to prevent rejection of allograft vascularized bone grafts include:

1) Alteration or elimination of graft alloantigens (immunomodulation),
II) Limitation of donor versus graft immunologic responses, and

III) Combination of I and II

1.8.1 I) Immunomodulation

In the clinical setting, most bone grafting is performed with autogenous tissue. Allograft bone remains a lesser substitute, largely due to the immune responses of the host that render these grafts at higher risk for complications including impaired or delayed union, stress fracture, and infection. The goal of graft immunomodulation would be to alter or eliminate the antigenic epitopes such that the allograft is no longer recognized as foreign by the recipient immune system. The ability to selectively perform this sort of manipulation through biological methods is not yet available. However, various physical, nonspecific manipulations have been used in the context of bone and cartilage allografts.

In attempts to reduce antigenicity, allograft bone has been subjected to freezing, lyophilization, boiling, chemical sterilization, irradiation, and antigen extraction. Although the immunogenicity of allograft bone may be markedly reduced by these measures, they render the graft devoid of all viable cells and able only to act as a passive framework for bony ingrowth and creeping substitution.

The advantage of vascularized bone grafts is that they maintain cellular viability and are able to actively participate in the bone healing process. Transplanted epiphyseal plates must also be vascularized if viability and predictable growth are to be maintained. Although acceptable for non-vascularized transfers of allograft bone, physical manipulations used to reduce antigenicity, such as freezing, would clearly be unsuitable for vascularized grafts.

Although antigens cannot be selectively altered to reduce immunogenicity at this time, efforts are routinely made in modern organ transplantation to match the MHC haplotypes of
donors with recipients such that the antigenic disparity between the two is minimized. Certain studies in allograft vascularized bone transplants have suggested a role for partial matching of the MHC between donors and recipients\textsuperscript{167}. Tissue matching is unlikely to be clinically practical for allograft transplants of musculoskeletal tissue. The candidates for such procedures are likely to be few, as are potential donors, thereby limiting the chance of finding successful matches. In addition, as in the case of allotransplantation of other organs, the patient would have to be continuously "on call" in case of a successful match. Demands of this nature may be difficult to justify for the transplantation of musculoskeletal tissue.

1.8.2 II) Systemic Immunosuppression

The second strategy, the suppression of the recipient's intrinsic immune system, is routinely invoked in modern transplant surgery with the use of immunosuppressant drugs. The discovery of Cyclosporine, a potent suppressor of the immune system, has revolutionized the field of solid organ transplantation. Like other current immunosuppressants, Cyclosporine is a non-specific suppressor that leaves the recipient susceptible to infections. Cyclosporine also has serious side-effects, including nephrotoxicity, hypertension, and neurotoxicity when taken for prolonged periods\textsuperscript{41,47}. In general, prevention of host versus graft responses requires administration of the drug for the lifetime of the recipient\textsuperscript{243}, usually in conjunction with other drugs such as prednisone and azathioprine. The risk to benefit ratio of long-term treatment with Cyclosporine is acceptable when used in context of a life-saving organ transplant. With musculoskeletal allografts, such as an epiphyseal plate transplant, the risk is not ethically justifiable.
1.8.3 III) Combined Strategies

A theoretical protocol to prevent rejection of vascularized epiphyseal plate transfers might consist of a combination of the above two strategies. Such a strategy would employ systemic immunosuppression for a short period while anticipating graft neovascularization by vessels of host origin and possibly a reduction in the immunogenic components of the donor tissue.

Short-term use of Cyclosporine has been advocated in the context of non-life-threatening conditions such as rheumatoid arthritis, dermatologic disorders and other inflammatory conditions. The use of Cyclosporine, or other immunosuppressants, may be ethically acceptable in the context of musculoskeletal transplantation if used only for a relatively short, finite period. Acceptance for the use of Cyclosporine in this manner is unlikely to be difficult to achieve.

The risk of short-term use of immunosuppressants following allograft organ transplantation is that graft rejection occurs upon withdrawal of the drug(s). This unfortunate consequence could potentially be avoided if some degree of antigen modulation was to occur in the graft (to become replaced by host antigens) and a new blood supply established (other than the donor pedicle). Bone tissue differs from renal and cardiac tissue due to its unique healing and regenerative properties. Theoretically, following transplantation of allograft bone, donor-origin bone and cellular material would undergo turnover to become replaced with like tissue of host origin. During the healing process, some immunogens may decay naturally, others may be removed by osteoclast activity. Persistent donor antigens may be sequestered secondarily by host apposition of new bone. Thereafter, the normal physiologic remodeling of the viable host bone will result in gradual turnover with time. The allografted marrow might also become repopulated by recipient
progenitor cells\textsuperscript{134}, eventually leading to a relatively higher degree of host-derived, rather than donor-derived tissue.

Vascular ingrowth from the recipient site bone would also occur as a normal consequence of the healing process. The vascular pedicle and microcirculation, as in renal and cardiac transplants, would be expected to retain their genetic identity as tissue of donor origin\textsuperscript{48, 166, 182}, and to undergo rejection following removal of immunosuppression. The newly acquired blood supply of recipient origin would ideally then supply the metabolic demands of the graft and epiphyseal plate. Although still of donor origin, the epiphyseal plate would be protected due to its privileged state. Because the epiphyseal plate is avascular, it receives nutrients by diffusion. The origin of the vessels from which these nutrients diffuse, whether donor or recipient, should not be important with respect to the physiological needs of the epiphyseal plate. With these developments, withdrawal of immunosuppression may be compatible with continued viability.

1.8.4 Short-term immunosuppression with vascularized osteochondral transfers

The special (self-renewal) characteristics of bone have prompted other investigators to attempt short-term immunosuppression following vascularized allograft transfer. Most of these studies have not specifically focussed on the behavior of the epiphyseal plate following transplantation. Although the emphasis was more on the viability of vascularized bone grafts following withdrawal of short-term immunosuppression, the results have important implications for this model because of the many shared characteristics with vascularized bone grafts.

Investigations of short-term immunosuppression in vascularized bone grafts have utilized several animal models. These can be classified into three groups based on the type of transfer performed:

I) Those transplanting the knee joint, or a component of it, to a heterotopic location
II) Those transplanting the knee joint, or a component of it, to an orthotopic location

III) Those transplanting an intercalary (segmental) bone graft to an orthotopic location

1.8.4.1 1) Knee joint or component transplants - Heterotopic locations

The relevant studies in this group were performed in rats using the whole-knee transplant model. The grafts were transferred to a subcutaneous position in the recipient’s abdomen and anastomosed to the reflected femoral vessels.

Investigators attempted to exploit the ability of bone grafts to undergo cellular turnover as a potential means to reduce their immunogenicity following transplantation. In two studies from the same laboratory, the effectiveness of this process was evaluated in an innovative manner. Using inbred rat strains, vascularized knee transplants were performed across a major histocompatibility barrier into immunosuppressed recipients. These grafts were then removed at various times post-transplant and transferred into naïve, non-immunosuppressed recipients syngeneic with the first host, and monitored for rejection. With this experimental design, the rapidity and extent of rejection would presumably be diminished in the second host had significant reductions in immunogenicity taken place while in the first host.

In the first study, four weeks of immunosuppression was given to the initial recipient prior to removal of the graft. The vascularized grafts rejected rapidly in the second host suggesting that little, if any, graft repopulation or antigen modulation had occurred. The authors postulated that the period of immunosuppression was insufficient to allow for significant antigen modulation. The second study was conducted with longer periods of “incubation” in the first recipient. Grafts were removed and transferred into the second, naïve host after 12 to 26 weeks of immunosuppression in the initial host. Regardless of the incubation time spent in the initial
host, all grafts showed evidence of rejection and epiphyseal plate death less than 4 weeks following transplantation into the second host. Thus, the host-anti-graft immune response was only slightly less aggressive than that seen in the previous study with 4 weeks of immunosuppression, and provided little evidence for repopulation of the graft with cellular components of donor origin.

Although the grafts in the latter investigation had spent over 3 months in the initial, immunosuppressed host, they were located in a subcutaneous, heterotopic recipient site. Thus, there was no possibility for graft neovascularization and creeping substitution, or local migration of host cells across the osteotomy site. For this reason, the authors advocated further investigations with orthotopic transplantation of the graft in order to establish bone contact between the vascularized transplant and the host.

1.8.4.2 II) Knee joint or component transplants - Orthotopic locations

Studies in this group performed orthotopic transplantation of the knee, or a component thereof, to a recipient immunosuppressed for a short-term period. Bone contact was established between the donor and recipient and stabilized with rigid internal fixation.

Results of orthotopic, whole-knee allografts in dogs were not encouraging. Recipients were immunosuppressed for 3-4 months after transplantation. Withdrawal of CsA led to rapid rejection of the donor tissue within 2-3 weeks, an interval that was little different from those without immunosuppression at all.

A similar model in rats demonstrated comparable results, although with a slower evolution. Recipients were immunosuppressed periods of two and four weeks. Withdrawal of CsA led to gradual rejection within 3 weeks, followed by pathological fractures and joint instability.
In an attempt to evaluate the long-term function of knee hemijoint transfers, investigators performed orthotopic transplantation of the distal femur including the distal femoral epiphysis. Recipients were immunosuppressed for 10 weeks and grafts were removed for analysis up to 12 months postoperatively. Although stable bone union was achieved by 8 weeks, rejection of the transplant ensued after withdrawal of CsA and was associated with elevated cell-mediated and humoral immune responses. Although bone contact was established with this model, investigators found relatively little evidence of remodeling or creeping substitution within the allograft itself.

The studies in this group provided relatively little support for short-term immunosuppression regimens. It should be noted, however, that in this experiment the opportunities for neovascularization and graft antigen modulation were not optimal. Graft designs that included an articular surface, such as the whole-knee or distal femur models, offered the possibility for revascularization of the epiphyseal plate from only the one site of bone contact with the host. Although revascularization of the metaphyseal side of the graft was possible, the epiphyseal circulation, vital for the viability of the resting and proliferative zones, remained un restored.

1.8.4.3 III) Intercalary bone grafts

With intercalary bone grafts, revascularization can potentially occur from both the proximal and distal sites of contact with the host. Bone allografts of this type with short-term immunosuppression were generally studied as a potential method for the reconstruction of large segmental defects of long bones, such as the tibia. Although the intercalary bone grafts did not contain an epiphyseal plate, they offer some insight into the behavior of bone allografts with the potential for revascularization from both ends. All investigations used a large segment of diaphyseal bone based on the nutrient artery.
In a study of revascularized intercalary tibial allografts in dogs, four weeks of Cyclosporine was administered following transplantation\(^3\). Upon sacrifice of the animals at 20 weeks postoperatively, evidence of remodeling was found in the grafts although the actual viability was not reported.

In a similar model\(^5\), immunosuppression was administered for 3 months after transplantation. Despite the longer immunosuppression interval, rapid rejection of the grafts occurred following withdrawal although union was maintained. Donor bone had been replaced with fresh bone at both sites of contact with the host.

Immunohistochemical techniques were used to determine the origin of osteocytes in rat intercalary tibial allografts\(^185\). Using a peroxidase-conjugated monoclonal antibody directed against the donor MHC, the fate of donor osteocytes was followed after short-term immunosuppression. Donor osteocytes survived as long as immunosuppression continued, and died rapidly thereafter. Nonetheless, recipient osteocytes had already invaded both ends of the allografted bone at the osteosyntheses.

In a subsequent rat study\(^186\), two weeks of immunosuppression was administered to recipients of intercalary tibio-fibular allografts. With only two weeks of immunosuppression, bone union was either delayed or unpredictable. The grafts showed decreased scintigram uptake 3 weeks after withdrawal, coupled with histological evidence of rejection within 2-3 weeks. Withdrawal of immunosuppression prior to completion of bony union likely contributed to the early demise of the allografts in this study.
1.8.5 Factors influencing the development of the current model

The studies with short-term immunosuppression discussed above focussed mainly on the behavior of vascularized bone and/or joint grafts, and relatively little on the function of epiphyseal plates. Nonetheless, they provide insight into features that may enhance survival of vascularized epiphyseal plate transplants following withdrawal of short-term immunosuppression.

Vascular ingrowth and remodeling of the allograft occurs first at the osteosyntheses, albeit relatively slowly. If this process is to occur to a significant degree, *immunosuppression must be continued for a period longer than that required for bone union.* In this regard, transplants with *two sites of bone contact* with the host, as in the intercalary grafts, have an advantage with respect to those models that include the articular surfaces. *Removal of the articular surface has theoretical value for epiphyseal plate grafts because bone union and neovascularization could occur on both the epiphyseal and metaphyseal sides of the epiphyseal plate.* In addition, the models discussed above all had contact only with cortical bone. The process of graft revascularization is faster with cancellous bone\(^{161}\), and therefore may be expedited by *transplantation of the allograft into a corticocancellous recipient site.*

Although complete cellular repopulation of a bone infarct occurs relatively rapidly\(^{49}\), host replacement of donor cells seems to occur much more slowly\(^{185}\) following allograft bone transplantation. Rather than actual replacement of donor cells by recipient cells, coexistence of the two seems more likely, at least within the short time periods studied so far. Thus, rejection of remaining cellular material of donor origin may be expected, even in a successful model for allograft vascularized epiphyseal plate transfers with short-term immunosuppression.
Given that the previous short-term studies were investigating solutions for massive bone and joint defects, the donor grafts were relatively large. These allografts contained high loads of bone and donor cellular material, and limited the amount of neovascularization and cellular repopulation possible within a relatively short period of immunosuppression. A more successful model would ideally consist of a very small amount of adjacent bone on both sides of the epiphyseal plate. An important goal in the graft design of the current study was to establish the smallest possible epiphyseal plate graft compatible with preserved viability following microvascular transplantation.
1.9 OBJECTIVES

The objectives of the experiments reported in this study were:

I) To develop a model for the vascularized transplantation of the proximal tibial epiphyseal plate of the immature rabbit, with a minimum amount of adjacent bone, to a recipient site in the iliac crest

II) To validate this model using histologic, morphometric and qualitative and quantitative techniques for the assessment of vascular perfusion

III) To compare the viability of vascularized allograft epiphyseal plate transplants, both with and without immunosuppression, using immunohistochemical techniques for the identification of S-phase nuclei

IV) To evaluate viability of vascularized allograft epiphyseal plate transplants with bone contact after withdrawal of short term immunosuppression, as compared to similar grafts without bone contact

1.10 HYPOTHESIS

A vascularized epiphyseal plate allograft with minimal adjacent bone will remain viable following withdrawal of short-term immunosuppression provided bone contact is maintained.
Chapter Two:

2 MATERIALS AND METHODS
2.1 ETHICS

Animal handling was conducted according to the animal care guidelines of the Hospital for Sick Children. All procedures and investigations were conducted following protocol approval by the Animal Care Committee.

2.2 MODEL DEVELOPMENT - VASCULAR AND ANATOMIC STUDIES

2.2.1 Demonstration of Vascular Anatomy by Corrosion Casting

2.2.1.1 Corrosion Casting of Intact Rabbit Hindlimbs

The vascular supply of the proximal tibial epiphyseal plate was studied in order to facilitate planning of the operative technique. Both hindlimbs of four rabbits were prepared for injection of methyl methacrylate (Batson’s Corrosion Casting Kit, Polysciences Inc). The legs were disarticulated through the hip joint and the soft tissues divided using electrocautery. The femoral artery and vein were cannulated with a 25-gauge angiocath, which was tied in place with 2-0 silk suture and then sealed with Super Glue. All blood was flushed from the limb by irrigation with isotonic saline. The methyl methacrylate was prepared and dyed according to the manufacturer’s instructions. It was injected into the femoral artery at a rate of 2 cc/min using a 10 cc syringe until the fine capillaries were seen to fill.

2.2.1.2 Corrosion Casting of Isolated Proximal Tibial Epiphyseal Plate

The casting procedure was also performed after isolation of the growth plate to confirm that the vessels supplying the graft had not been compromised or eliminated by the surgical dissection process. The growth plate of the proximal tibia was isolated on the popliteal pedicle in a manner identical to that performed in the operative procedure, described below. Meticulous hemostasis
was obtained to prevent leakage of the casting fluid. The animal was then killed and vessel preparation and injection was performed as outlined above.

The soft tissues were dissolved using a concentrated base solution (Maceration solution, Polysciences Inc). The remaining structures were rinsed in tap water and allowed to dry by air.

2.2.2 Investigation of Graft Perfusion

2.2.2.1 Quantitative analysis - Radioactive microspheres

Injection of radioactive microspheres was used to quantify graft perfusion. This technique has been used extensively in our facility and has been described for use in pigs\textsuperscript{164} and rabbits\textsuperscript{76}.

Arterial access was obtained at three sites in preparation for the microsphere injection. Arteries were cannulated with 5-French polyethylene tubing and flushed with heparinized saline. The right brachial artery was used for monitoring of blood pressure using an electronic blood pressure monitor (Model 78304A, Hewlett-Packard). During the microsphere injection and for 30 seconds afterward, blood was withdrawn from the left femoral artery at a flow rate of 2.06 ml/min using a Harvard Apparatus infusion/withdrawal pump. \textsuperscript{57}Cobalt microspheres with a diameter of 15 microns (NEN-trac microspheres, NEM-012A, Mandel Scientific, Guelph) were suspended in a 5% sucrose solution. These were administered at a dose of 200,000 spheres/kg and injected into the left ventricle via a catheter in the left carotid artery after vigorous sonication and agitation.

Twelve rabbits were divided into three equal groups based on the extent of isolation of the graft:
Group 1 - Unmanipulated vasculature. The right proximal tibia was left unaltered prior to injection of microspheres. The graft was removed in the usual manner after sacrifice of the rabbit.

Group 2 - Vascular island. The graft was isolated on the arteriovenous vascular pedicle and allowed to perfuse for 1 hour, at which time the microspheres were injected.

Group 3 - Free tissue transfer. The complete allograft epiphyseal plate transplant operative procedure was performed. Forty-eight hours afterward, microspheres were injected and the specimen removed. Animals received immunosuppression with Cyclosporine 10 mg/kg/day SC during this interval.

The animal was sacrificed following the microsphere injection and specimens were then obtained. These included the epiphyseal plate graft, the right iliac wing, and both the right and left kidneys to compare the extent of mixing of the microspheres. In each case, the skeletal tissue was cleared of all soft tissue and periosteum. Radioactivity of the specimens was analyzed in a gamma scintillation counter (Beckman, Model 8000).

Bloodflow in the epiphyseal plate grafts was calculated using standard formulae\textsuperscript{164} and compared between groups using 1-way ANOVA.

\section*{2.2.2.2 Qualitative Analysis – India Ink and Barium Injections}

Steps were taken to evaluate the distribution of bloodflow within the epiphyseal plate graft. Following isolation of the graft on its vascular pedicle, 1.5 cc of India ink was injected into the popliteal artery over a period of 60 seconds. This technique allows subsequent histological visualization of the vascular beds being actively perfused\textsuperscript{54,137}. Injections were performed in one graft 1 hour after dissection from the donor, and in two grafts 6 weeks after transplantation.
into the iliac crest of a continuously immunosuppressed recipient rabbit. The same protocol was repeated in an additional set of animals but with the use of barium sulphate instead of India ink.

In each case, the grafts were submitted for fine-detail radiography and routine histological processing in a manner identical to the specimens in the principal experiment.

2.3 OPERATIVE PROCEDURE

2.3.1 Experimental Animals

Pathogen-free, female, New Zealand White rabbits were used for all experiments. All animals were obtained from a single supplier (Charles River Laboratories, Quebec) that maintains a large, outbred colony. The rabbits arrived at our facility at ten weeks of age in three non-sibling pairs per week. Each non-sibling pair consisted of one donor and one recipient. Surgery was performed on the three pairs within one week of arrival.

2.3.2 Anesthesia

Sedation of the rabbits was achieved with acepromazine maleate (Atravet, Ayerst Laboratories, Montreal) 2 mg/kg IM administered 15 minutes prior to induction. The appropriate areas of the rabbits were then shaved.

A contoured facemask was fitted over the rabbits’ snout and fixed in place with masking tape. A mixture of 50/50 nitrous oxide and oxygen with 2.5% Halothane was administered via a Bain circuit until anesthesia was achieved (approximately 10 minutes). These settings were usually sufficient for the duration of the procedure. The level of Halothane was increased in small increments if the rabbit showed signs of stimulation during the operative procedure. Intubation was not performed.
Core temperature was maintained during the operation with a hyperthermia blanket. Ringer’s lactate was infused through a marginal ear vein at a rate of 10-15 ml/kg/h. The flow rate was adjusted using a Dial-A-Flow intravenous flow control extension set (Abbott Laboratories, Chicago).

2.3.3 Donor Rabbit - Isolation of Graft

The rabbit was positioned supine and the right lower extremity prepped with isopropyl alcohol followed by Betadine. The foot was wrapped with a sterile towel after which the area was draped such that only the right lower extremity was exposed (FIGURE 2:, pg. 49).

A linear incision was made on the medial aspect of the right leg from the groin to the distal tibia. Skin flaps were elevated and the femoral vessels isolated. The deep fascia was incised parallel to the saphenous neurovascular bundle beginning in the distal tibial region and carried proximally to expose the descending geniculate vessels. These, as well as the saphenous artery and vein, were isolated, cauterized and divided. The popliteal vessels were exposed by division of the overlying musculature, including the thigh adductors and the medial head of the gastrocnemius (FIGURE 3:, pg. 50).

The deep fascia over the anterior tibia was elevated off the bone and reflected laterally in continuity with the patella, quadriceps and biceps femoris following division of the patellar tendon. This process required elevation of the quadriceps off the shaft of the femur. The lateral head of the gastrocnemius was divided below the gastrocnemius sesamoid. At this point, the entire length of the popliteal vessels became visible. Vascular branches off the popliteal vessels, including the caudal femoral artery and vein, were isolated, cauterized and divided.

The anterior tibial musculature was divided at the level of the mid-tibia and reflected superiorly allowing cauterization and division of the anterior tibial vessels as they came into
view. The muscles over the lateral aspect of the proximal tibia were removed with care to leave a muscle cuff and the periosteum intact.

The femur was divided using a reciprocating saw (Howmedica Chirodrill, Germany) at a level 2.0 cm proximal to the knee joint, taking care to protect the femoral vessels. With the distal femur and tibia mobile, the femoral vessels were dissected free of any remaining attachments to muscle. The tibia was then placed with the lateral side down, and the distal femur dissected off the vascular pedicle. Division of the menisci and cruciate ligaments completed the removal of the distal femur.

Using a high-speed burr, the articular cartilage of the tibial plateau was removed down to bleeding subchondral bone. The tibial plateau was rendered completely flat, as judged by comparison to a ruled edge, to facilitate accurate fitting into the recipient site. Removal of the tibial tuberosity was accomplished with bone shears. This maneuver allowed visualization of the anterior edge of the epiphyseal plate cartilage. The osteotomy on the metaphyseal side of the epiphyseal plate was made 1 mm distal to this anterior edge after performing a subperiosteal dissection of the tibia up to this level (FIGURE 4:, pg. 51). Extreme care was taken not to traumatize the caudal tibial vessels during the osteotomy, as they were located immediately behind the cortex of the posterior tibia. Upon division of the fibula 1 cm distal to the tibiofibular joint, isolation of the proximal tibial epiphyseal plate on its vascular pedicle was complete. A template was made against the lateral profile of the graft to allow accurate sizing of the recipient site. The graft was covered with saline-moistened gauze and allowed to perfuse while the recipient site was prepared.
FIGURE 2: Diagram showing positioning of donor rabbit. Major vessels of the lower extremity are shown in relation to skeletal structures.

Legend:
A - Position of donor rabbit showing incision placement
B - Distal femur
C - Proximal tibia
D - Femoral vessels
E - Popliteal vessels (graft vascular pedicle)
F - Saphenous vessels
FIGURE 3: Diagram showing exposure of vascular pedicle in donor rabbit.

Legend:
A - Quadriceps muscles
B - Gracilis muscle (cut and reflected)
C - Medial head of gastrocnemius (cut and reflected)
D - Femoral vessels
E - Popliteal vessels (graft vascular pedicle) with forceps grasping adventitia
F - Saphenous vessels (ligated and transected)
FIGURE 4: Diagram showing operative procedure for removal of proximal tibial epiphyseal plate graft, with vascular pedicle intact, from donor rabbit.

Legend:
A - Removal of articular cartilage using high-speed burr
B - Anterior edge of proximal tibial epiphyseal plate
C - Cut edge of quadriceps muscles
D - Osteotomy performed 1-2 mm inferior to anterior edge of epiphyseal plate using reciprocating saw
E - Undivided vascular pedicle (popliteal vessels) perfusing epiphyseal plate graft
F - Reciprocating saw
FIGURE 5: Diagram showing positioning of recipient rabbit. Incision placement is shown in relation to underlying skeletal structures.

Legend:
A - Position of recipient rabbit showing incision placement
B - Incision edge
C - Iliac crest
D - Wing of ilium with overlying abductor musculature (shaded)
E - Femur
F - Lower vertebral column
FIGURE 6: Diagram showing operative procedure for insertion, fixation, and revascularization of allograft epiphysal plate transplant in recipient rabbit.

Legend:
A - Epiphysal plate graft removed from donor rabbit
B - Insertion of graft into defect in recipient iliac crest
C - Transfixion of graft with two 0.7 mm K-wires
D - Isolation and preparation of recipient vessels
E - Microanastomosis of donor and recipient vessels
2.3.4 Recipient Rabbit - Insertion of Graft

The recipient rabbit was sedated and induced in an identical manner to the donor and then positioned in the left-lateral position. A curvilinear incision was made over the iliac crest and carried down through the fascia (FIGURE 5:, pg. 52). The superior margin of the tensor fascia lata was identified. A plane was developed between this muscle and the adjacent fat pad. At this point, the recipient vessels, the deep circumflex iliac artery and vein, could be seen within the fatty layer ascending anterior to the iliac spines. These vessels were dissected free, clamped proximally with a microvascular clamp, and divided distally. The iliac crest was exposed with electrocautery. The abductor muscles of the thigh were then stripped off the lateral surface of the ilium with a periosteal elevator.

2.3.4.1 Groups with Bone Contact and Skeletal Fixation

The bony recipient site was then prepared. The template of the donor graft was used as a guide to cut a defect of matched-size in the iliac crest. This defect was cut using a reciprocating saw with a 5 mm blade, was rectangular in shape and located in the central (thickest) portion of the iliac wing (FIGURE 6:, pg. 53). The donor epiphyseal plate graft was removed from the rabbit following heparinization with 250 U heparin IV (Hepalean). The graft was then inset into the recipient defect. Minor adjustments in the size of the defect were performed as necessary using a high-speed burr to ensure a snug press-fit of the graft in place. Fixation of the graft was achieved with two unthreaded 0.7 mm K-wires (Zimmer, Mississauga) which were driven longitudinally through the iliac wing from posterior to anterior, thus transfixing the graft perpendicular to the epiphyseal plate (FIGURE 6:, pg. 53).
2.3.4.2 Groups without Bone Contact or Fixation

The ilium was exposed as above, although no bone cuts were performed. The graft was placed with the epiphyseal surface adjacent to the ilium, after placing it within a latex rubber chamber from which only the pedicle exited. To control for the effects of fixation, two 0.7mm K wires were driven through the epiphyseal plate in the same position they would have occupied had the graft been situated in the iliac crest recipient site.

2.3.4.3 Microanastomoses and Closure

The donor and previously prepared recipient vessels were cut to approximate without tension. After stabilization in a double microvascular clamp, the vessels were anastomosed in an end-to-end fashion using standard technique with approximately six to eight 10-0 nylon sutures (Davis and Geck, Manati) per vessel. The veins and donor artery were approximately 1.0 mm in diameter. The recipient artery was approximately 0.6 mm in diameter. After removal of all clamps, the vessels were observed for restoration of bloodflow to the graft. Graft bleeding was evaluated and recorded and the venous outflow-patency test was performed. The wound was closed over the graft in layers using continuous running sutures of 3-0 Dexon (Davis and Geck, Manati).

2.3.4.4 Contralateral Unmanipulated Proximal Tibia

A screw was placed in the mid-diaphysis of the left tibia as a marker for growth of the contralateral proximal tibial epiphyseal plate. Standardized teleoroentgenograms of the left tibia were obtained postoperatively in a manner identical to previous investigations at this institution\(^3\). This technique has been shown a reliable and valid method for assessment of proximal tibial epiphyseal plate growth in the rabbit\(^8\).
2.3.5 Postoperative Care

Rabbits were administered long-acting penicillin (PenLong, 0.5 cc IM) and analgesia (Temgesic, 0.5 cc IM). Sterile dry dressings were applied.

All rabbits were allowed to move about their cages freely, and were fed a standard diet of rabbit food pellets and bottled water. Dressings were removed after healing of the operative site was complete, approximately two weeks postoperatively.

2.4 MODEL VALIDATION - PRELIMINARY EXPERIMENTS

2.4.1 Study A - Investigation of bony union

This study investigated the time required for bone union between the donor epiphyseal plate graft and the recipient site bone. The complete transplant procedure with graft fixation, described above, was performed in 10 animals. Animals were sacrificed at 7-day intervals beginning 10 days after surgery. Specimens were removed and analysed radiographically and histologically. Emphasis was placed on evaluation of the extent of bony union of the epiphyseal plate graft with the iliac crest recipient site.

2.4.2 Study B - Graft survival and efficacy of immunosuppression

The purpose of this study was to evaluate epiphyseal plate viability both with and without continuous immunosuppression, in epiphyseal plate grafts both with and without bone contact. In immunosuppressed groups with bone contact, animals were kept for periods of 6 weeks (n = 5) and 10 weeks (n = 4) following surgery. Animals without immunosuppression were kept for only 6 weeks prior to sacrifice. Immunosuppression was provided by Cyclosporine 10 mg/kg/day SC in designated animals. Animal allocations are summarized in TABLE 2: (pg. 57).
In an additional group of five animals, transplantation of the epiphyseal plate graft was performed without microvascular anastomosis. These ischemic controls received immunosuppression for 6 weeks.

Specimen removal and processing for all specimens was conducted as described below.

**TABLE 2:** Preliminary Study B Design
Continuous immunosuppression

<table>
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<td>n = 5</td>
</tr>
</tbody>
</table>
2.5 PRINCIPAL EXPERIMENT DESIGN: Short-term immunosuppression

2.5.1 Sample size calculations

Sample size calculations were performed in collaboration with a biostatistician. The acceptable error limits were set to $\alpha = 0.05$ and $\beta = 0.1$. The BrdU labeling index was chosen as the main outcome measure. In order to detect a difference of 70% between experimental and control epiphyseal plates, the minimum size was calculated as $n = 11$ animals per group. A seventy-percent difference in labeling index was chosen, as it is approximately twice that which might be expected in normal animals at the age in question\textsuperscript{73, 74}. To account for possible animal losses due to death or technical failure, 12 animals were allocated to each group.

2.5.2 Experimental and control groups

Animals were randomly allocated to each of the four main groups (TABLE 3:, pg. 60). A break in the randomization protocol was required, however, due to poor efficacy of the original immunosuppression regimen. The consequence of this altered protocol was execution of the experiment in two segments. The first involved random allocation of non-immunosuppressed animals to groups with or without bone contact, followed by the second segment where animals were immunosuppressed with CsA, and again randomly allocated to groups with or without fixation. The events leading to the break in full randomization are discussed in detail in APPENDIX C: (pg. 120).

Experimental group 1A ("Fix\textsuperscript{*}CsA"): Animals received allograft microvascular growth plate transfers to the iliac crest recipient site with bony fixation. The immunosuppression protocol consisted of 6 weeks of continuous therapy with Cyclosporine, followed by 4
weeks without. A metal screw was placed in the tibial diaphysis of the left, 
unmanipulated tibia for measurement of growth.

Experimental group 2A ("Fix⁰CsA"): Transplants were performed as in Group 1A, but no 
immunosuppression was administered. Animals were kept for only 4 weeks following 
surgery, such that the period without immunosuppression would match those in the 
short-term group. A metal screw was placed in the tibial diaphysis of the left, 
unmanipulated tibia for measurement of growth.

Experimental group 1B ("Fix⁰CsA"): Animals received allograft microvascular growth plate 
transfers to a soft-tissue pocket without bony fixation. Short-term immunosuppression 
consisted of 6 weeks of continuous therapy with Cyclosporine, followed by 4 weeks 
without. A metal screw was placed in the tibial diaphysis of the left, unmanipulated tibia 
for measurement of growth.

Experimental group 2B ("Fix⁰CsA"): Transplants were performed without fixation as in group 
1B, but no immunosuppression was administered. Animals were kept for 4 weeks 
following surgery. A metal screw was placed in the tibial diaphysis of the left, 
unmanipulated tibia for measurement of growth.
### TABLE 3: Principal Experiment Design

**Short-term immunosuppression**

<table>
<thead>
<tr>
<th>SHORT-TERM IMMUNOSUPPRESSION</th>
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<td>CONTACT</td>
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<tr>
<td></td>
<td>Experimental Group 1A (n = 12)</td>
<td>Experimental Group 2A (n = 12)</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Experimental Group 1B (n = 12)</td>
<td>Experimental Group 2B (n = 12)</td>
</tr>
</tbody>
</table>

### 2.6 PREPARATION AND ADMINISTRATION OF IMMUNOSUPPRESSANT

Cyclosporine A (Neoral™ oral preparation) was used as the immunosuppressive agent. This drug was a generous gift of Novartis Pharmaceuticals (Dorval, Quebec). It was administered at a dosage of 10 mg/kg/day by subcutaneous injection. This route of delivery and dosage has been successfully used at our facility in the past.

The drug preparation was kept at room temperature and protected from light, according to the manufacturer’s instructions.

### 2.7 ADMINISTRATION OF FLUOROCHROME LABELS

Oxytetracycline (MTC Pharmaceuticals, Cambridge) was administered to rabbits 3 days postoperatively at a dose of 30 mg/kg IM. A second fluorochrome, DCAF (ICN Biomedicals) 75 mg/kg IP was given at the cessation of immunosuppression (Experimental group 1, Control group A) after dissolution in 0.5N KOH. DCAF was not administered to animals in
preliminary studies receiving continuous immunosuppression. The final label, xlenol orange (Sigma Chemical Company) 100 mg/kg IV\textsuperscript{176}, was given to all animals 1 hour prior to sacrifice (FIGURE 7:).

**FIGURE 7:** Experimental timeline for groups with short-term immunosuppression

<table>
<thead>
<tr>
<th>INTERVENTION:</th>
<th>Surgical procedure</th>
<th>Cyclosporine withdrawal</th>
<th>Animal sacrifice</th>
</tr>
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<tbody>
<tr>
<td>WEEKS:</td>
<td>-1 0 1 2 3 4 5 6 7 8 9 10</td>
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</table>

**INJECTED LABELS:**
1. Xlenol orange
2. BrdU

2.8 SPECIMEN PREPARATION

2.8.1 Animal Sacrifice

Bromodeoxyuridine was administered to all rabbits to facilitate the identification of proliferating epiphyseal plate chondrocytes. One hour prior to sacrifice, the rabbit was given an intravenous injection of bromodeoxyuridine (Sigma Chemical Co.) 40 mg/kg IV dissolved in isotonic saline\textsuperscript{12}. Given that there are significant diurnal rhythms in the timing of DNA synthesis within the epiphyseal plate\textsuperscript{207}, all injections of BrdU were performed at a standardized time-of-day (9:00 am). The animal was then anesthetized in the usual manner. The vascular
pedicle to the graft was assessed using the operating microscope. Clinical tests of vascular patency, including the vessel outflow test and the uplift test were performed. Patency was further evaluated by observing for bleeding following partial transection of the vein on the host-side of the anastomosis, and then partial transection of the artery on the graft-side of the anastomosis. The vessels were considered patent if all of the above tests were positive. The rabbit was sacrificed by injection of pentobarbital (Euthanal, MTC Pharmaceuticals, Cambridge) into a marginal ear vein. Both the right iliac crest and left proximal tibia were harvested and were fixed in 70% ethanol. Specimens were stored at 4 degrees Celsius prior to processing.

2.8.2 Radiography

Standardized, lateral teleoroentgenograms of the left, unmanipulated proximal tibia were made in a manner identical to those performed post-operatively. All radiographs of specimens were converted into digital images using a flatbed scanner (Scanjet 4C, Hewlett-Packard) with a transillumination adapter attached to a microcomputer (Macintosh 7300, Apple Computer Co., Cupertino). Images were then analyzed on-screen using scientific image-analysis software (NIH Image, National Institutes of Health, Bethesda). The longitudinal dimension (epiphyseal plate transplants), or the epiphyseal plate-to-marker distance (unmanipulated tibias) was measured after appropriate calibration of the imaging software. These post-sacrifice measurements were compared to the immediate post-operative values to yield the extent of epiphyseal plate growth during the postoperative period.

2.8.3 Histological Processing

The control specimens were bisected in the sagittal plane down the midline of the tibia, through the intercondylar eminence. The specimens fixed in the iliac crest were divided in the
exact same plane; identification of the appropriate location was straightforward based on external
landmarks of the graft (FIGURE 8.; pg. 64). The medial and lateral halves of each specimen
were then cut into 5 mm thick sections. Each of these sections received fine-detail radiography
and were evaluated as described above (Section 2.8.2). One half was decalcified for histology
and immunohistochemistry, the other half remained undecalcified for interpretation of
fluorochrome labels.

After decalcification of the tissue in formic acid, the specimens were embedded in paraffin.
Sections of 4 µm thickness were made in the sagittal plane. Two sections were prepared for
staining with a WHO stain (hematoxylin, saffron, phloxin, Alcian green), and two were left
unstained for immunohistochemistry.

For the undecalcified tissue, sections were prepared after embedding in plastic and grinding
to a thickness of 250 µm.

2.8.4 Immunohistochemistry

The decalcified, unstained, paraffin embedded tissue sections were processed for
immunohistochemical detection of BrdU. The technique was modified from that described by
Apte\textsuperscript{12}, and is described in detail in APPENDIX B: (pg. 118). Slides were de-paraffinized in
toluene and then rehydrated in a graded series of ethanols. Nuclease digestion was performed by
incubation in a 0.4% pepsin solution at 42°C for 30 minutes. The slides were rinsed and then
incubated with the primary antibody (mouse anti-BrdU) in a moist chamber for 16 hours. This
was followed by further incubations with the linking antibody (biotinylated goat-anti-mouse IgG)
and the peroxidase (streptavidin enzyme complex) for 30 minutes each. Finally, slides were
counterstained with Mayer’s hematoxylin.
FIGURE 8: Radiographs showing plane of bisection of ilium and epiphyseal plate graft, 6-weeks post-transplantation.

Legend:

A - Lateral radiograph of ilium showing plane of specimen cross-section

B - Radiograph of specimen cross-section (5mm thick)

1 - margins of epiphyseal plate graft
2 - epiphyseal plate
A series of positive and negative controls were used to validate the immunohistochemical protocol. Histological sections of paraffin-embedded gut epithelium, a tissue known to exhibit strong positive uptake of BrdU, were processed alongside the experimental specimens. Additional positive controls included sections from the unmanipulated proximal tibial epiphyseal plate from each recipient rabbit. Negative controls included proximal tibial epiphyseal plates from rabbits that had not received BrdU and sections where the anti-BrdU antibody was withheld from the processing regimen.

2.8.5 Microscopy and Histomorphometry

The WHO-stained sections were used to evaluate histological features of the grafts using a light microscope. The sectioned and mounted growth plates were divided into peripheral and central sections, corresponding to the regions of the growth plate supplied (in the normal state) by the metaphyseal and periosteal circulations. General histological features were documented directly from the microscopic slides in each of these areas. Two adjacent sections from each graft were analysed. A rejection index was calculated for each specimen based on published criteria\textsuperscript{168} (TABLE 1: pg. 30). The percentage of each epiphyseal plate that had closed was calculated, as was the extent of bony union (complete / incomplete) at the junction of the graft and the recipient site. The metaphyseal trabeculae were assessed qualitatively in comparison to the unmanipulated, control proximal tibia specimens. Using the appropriate microscopic filters (UV barrier filter K350, 2B), the presence or absence of the various fluorochrome labels was established.

The BrdU labeling index was calculated at two locations on each of two serial sections. At a magnification of 250X, all cells within the proliferative zone were identified according to morphological characteristics\textsuperscript{73} and counted. The number of positively-labeled nuclear profiles
within this zone were then counted and expressed as a percentage of the total number of
proliferative zone cells.

2.9 STATISTICS

Quantitative data was subjected to analysis using a statistical software package (Sigma Stat,
Jandel Scientific). The presence of treatment effects was determined using two-way ANOVA
with immunosuppression status and bone contact as the factors. Differences were considered
statistically significant for values of $p \leq 0.05$. Multiple comparison tests (Bonferroni) were then
used to compare means between groups if a significant treatment effect was found.
Chapter Three:

3 RESULTS
3.1 MODEL DEVELOPMENT

3.1.1 Vascular studies - corrosion casts

Following the intravascular injection of methyl methacrylate, the removal of soft tissues yielded the skeletal structures of the leg with the attached casts of the arterial and venous network. The arterial anatomy of the rabbit lower limb was previously described in detail by several investigators\(^{37,218}\) and our findings were compatible with these earlier reports. The particular value of the corrosion casts was that they provided a three-dimensional view of anatomic relationships. This facilitated the development of a surgical procedure compatible both with preservation of the delicate epiphyseal plate vascular supply and its removal with a minimal amount of adjacent bone.

Just proximal to the knee, the femoral artery bifurcated into the saphenous and the popliteal arteries. The saphenous traveled distally to provide the major arterial supply to the foot and the popliteal vessels continued into the popliteal fossa. Branches derived from the popliteal artery supplied the musculature of the lower leg, including the gastrocnemias, via the caudal tibial artery. The vascular anatomy of the popliteal fossa itself was most relevant to this project. Within an area extending 4mm above and below the proximal tibial epiphyseal plate, numerous small branches arose from the popliteal artery and its divisions, the posterior and anterior tibial arteries. These small branches supplied the medial and lateral epiphyseal condylar regions (inferior medial and lateral genicular arteries) and the central epiphysis through an epiphyseal vessel entering in the intercondylar fossa. The proximal metaphyseal area was supplied in the posterior aspect by a network of small metaphyseal arteries and fine periosteal vessels arising from the posterior and anterior tibial arteries (FIGURE 9; pg. 69). A similar periosteal network
FIGURE 9: Popliteal region of rabbit lower limb after injection of femoral artery with methyl methacrylate and removal of soft tissues

Legend:

A - Popliteal artery  
B - Caudal femoral artery  
C - Epiphyseal vessels  
D - Location of proximal tibial epiphyseal plate  
E - Metaphyseal vessels  
F - Posterior tibial artery  
G - Fibula
existed anteriorly; it was supplied on the medial side largely by the inferior medial genicular artery and laterally by the anterior tibial recurrent artery after it emerged from the tibio-fibular interosseous space. It was evident from these corrosion casts that with preservation of the periosteum and a thin muscle cuff, an osteotomy 1-2 mm inferior to the anterior edge of the epiphyseal plate would maintain the epiphyseal blood supply as well as the periosteal network to the proximal metaphysis. The nutrient artery, entering in the tibial diaphysis, would be excluded by such a procedure. This observation was confirmed by corrosion casts performed following isolation of the proximal tibial epiphyseal plate on its vascular pedicle.

3.1.2 Investigation of graft perfusion

3.1.2.1 Quantitative analysis - Radioactive microspheres

The mean graft bloodflow as calculated by the radioactive microsphere technique is listed below and represented graphically (FIGURE 10.; pg. 71). Values are in units of total bloodflow (ml/min) per 100g of tissue. Perfusion to grafts in Group 3 was assessed 48 hours after transfer into an immunosuppressed recipient. The complete data are provided in APPENDIX A: (pg. 117).

Group 1: Unmanipulated proximal tibia  Mean = 5.288  SD = 1.056  n = 4
Group 2: Island graft  Mean = 6.011  SD = 2.267  n = 4
Group 3: Free tissue transfer  Mean = 7.462  SD = 2.806  n = 4

The difference between these mean values was not statistically significant as determined by 1-way ANOVA (p > 0.05).
FIGURE 10: Perfusion of vascularized epiphyseal plate grafts
Quantification by radioactive microsphere injection

Mean bloodflow of proximal tibial epiphyseal plate grafts with different degrees of vascular isolation from the donor. There is no significant difference in the mean values between the three groups (P>0.05)

3.1.2.2 Qualitative Analysis

3.1.2.2.1 Island Grafts - 1 hour post-surgery

Injection of India ink into the popliteal artery of the island epiphyseal plate graft resulted in immediate blackening of the entire construct. Both the epiphyseal and metaphyseal areas of the
graft were evenly affected. Histological sections revealed particles of ink in both the epiphyseal and metaphyseal capillaries.

3.1.2.2 Immunosuppressed grafts with skeletal fixation - 6 weeks post-surgery

The vascular pedicles of the exposed grafts were patent according to clinical tests. Injection of India ink into the artery caused blackening of the entire graft, including the epiphyseal and metaphyseal regions. Additionally, the entire recipient iliac wing, on both the epiphyseal and metaphyseal sides of the graft, was blackened as well (FIGURE 11, pg. 73).

The appearance of the histological sections was similar to those of the island grafts. All areas of the tissue section were occupied by capillaries containing ink particles. Of most significance, metaphyseal capillaries invading the epiphyseal plate as well as those of the epiphyseal bone plate were clearly filled with ink particles (FIGURE 12, pg. 74).

Fine-detail radiographs of specimen cross-sections revealed evidence of barium in the epiphyseal and metaphyseal regions. These findings manifested in the form of radio-opaque "blushes", and corroborated the findings of the India ink studies.

3.2 MODEL VALIDATION - PRELIMINARY STUDIES

3.2.1 Study A - Investigation of bony union

Prior to conducting the principal experiment with short-term immunosuppression, it was necessary to determine the approximate time of bony union between the epiphyseal plate transplant and the recipient iliac crest. This information then allowed construction of an experimental timeline that used immunosuppression of sufficient duration to permit bony union and neovascularization.
FIGURE 11: Photographs of gross specimens after removal. Epiphyseal plate growth was seen only in immunosuppressed specimens. Injection of ink into the vascular pedicle of immunosuppressed specimens resulted in uniform blackening of graft and adjacent ilium.

Legend:

Arrows indicate graft dimensions
A - Comparison of immunosuppressed and non-suppressed specimens
B - Continuous immunosuppression for 6 weeks

1 - non-suppressed
2 - immunosuppressed
3 - unmanipulated ilium
1 - with ink injection
2 - without ink injection
**Figure 12:** Photomicrographs of transplanted epiphyseal plate following 6 weeks of continuous immunosuppression. India ink was injected into the vascular pedicle immediately prior to sacrifice, and can be seen in both the epiphyseal and metaphyseal capillaries.

Legend:
- A - Central epiphyseal plate
- B - Epiphyseal bone plate
- C - Metaphyseal capillaries
- 1 - India ink particles within epiphyseal plate microvasculature
FIGURE 13: Photomicrographs of juncture of donor and recipient bone edges at 10 and 17 days post-transplant showing progression of bony union.

Legend:
A - 10 days post-transplant
B - 17 days post-transplant
1 Donor bone (metaphysis)
2Recipient bone (ilium)
3Juncture of donor and recipient

Safranin O stain, 100X
Ten days after transplantation, the margins of the graft and recipient site were still well demarcated radiographically. This demarcation became less apparent at days 17 and beyond. These findings were clarified by the histological results. At day 10, a narrow gap was still visible between the bony margins of the graft and the recipient site. At day 17, gaps were all but obliterated, and there was clear evidence of bony integration (FIGURE 13: pg. 75). In all specimens examined from days 28 and beyond, bony union was complete. The margins of the original graft could still be identified, however, largely based on trabecular density and orientation.

3.2.2 Study B - Graft survival and efficacy of immunosuppression

Immunosuppression was administered for a 6-week interval postoperatively. Preliminary study A demonstrated that bony union was completed well within this time. The purpose of Preliminary study B was to establish the viability of the transplanted epiphyseal plates following continuous immunosuppression.

3.2.2.1 Gross findings

The vascular pedicles to the epiphyseal plate transplants were surrounded by fine adhesions to the surrounding tissue. In the immunosuppressed animals, the pedicle was easily exposed and revealed a pulsatile artery. All clinical tests of vascular patency were positive in this group of animals. In contrast, the vascular pedicles in the non-immunosuppressed animals were generally more difficult to expose due to a thick layer of fibrous scar tissue. The pedicles themselves were thin, non-pulsatile, atrophic fibrous strands and were invariably non-patent.

Upon removal of the specimens, the graft margins were visible but solidly incorporated with the bone of the iliac crest in both immunosuppressed and non-suppressed grafts. Only the
immunosuppressed specimens, however, showed evidence of increased longitudinal dimension compared to the original graft size.

3.2.2.2 Radiographic findings

In both immunosuppressed and non-suppressed grafts, fine-detail radiographs showed evidence of complete bony union between the donor and recipient-site bone. Radiographs of the 5-mm thick specimen cross-sections were the most informative. In these, the lucent band of the epiphyseal plate was easily seen, as were the locations of the graft / host interface. The ability to identify the graft / host interface facilitated the measurement of graft dimensions.

Although bony union had occurred in both groups, marked differences were otherwise evident in the radiographic appearance of the suppressed and non-suppressed specimens. The main differences were the amount of longitudinal growth and the degree of epiphyseal plate closure (TABLE 4; pg. 85).

The mean growth of the immunosuppressed epiphyseal plate grafts was 5.25mm ± 1.53mm. In contrast, the non-suppressed grafts provided no quantitative or qualitative evidence of growth. The measured change in dimensions of 0.056mm ± 0.103mm is likely attributable to error introduced by the measurement process.

Partial epiphyseal plate closure had occurred in all of the immunosuppressed but in none of the non-suppressed specimens. Closure was visible on the radiographs (FIGURE 14; pg. 78), but was specifically quantified from the histological specimens for greater precision (see below).

All immunosuppressed grafts demonstrated osteopenia in the metaphyseal area. In most cases, the metaphyseal columns were either shortened or absent altogether, leaving only a thin margin of bone inferior to the epiphyseal plate (FIGURE 14;). The non-suppressed grafts
FIGURE 14: Transplanted epiphyseal plate with bony fixation and 6 weeks of continuous immunosuppression. Closure of approximately 30% of the epiphyseal plate has occurred.

Legend:
A - Radiograph of graft with partial epiphyseal plate closure
B -Photomicrograph showing junction of closed and open sections of epiphyseal plate

WHO stain, 40X
had an appearance closer to that of the unmanipulated tibias, with relatively little evidence of metaphyseal osteopenia.

3.2.2.3 **Histological findings: non-immunosuppressed specimens**

Grafts without immunosuppression were consistently non-viable. Most of the epiphyseal plate lacunae were empty. Chondrocytes, when present, were eosinophilic and shrunken. Matrix mucopolysaccharides showed diminished uptake of the Alcian green component of the WHO stain. Instead of the bright green staining of the normal epiphyseal plate matrix, the non-suppressed grafts were pale and eosinophilic (FIGURE 15; pg. 80).

The epiphyseal plates were thickened due to enlarged hypertrophic zones. Chondrocytes were somewhat disorganized, but still arranged in longitudinal columns. The architecture of the non-immunosuppressed epiphyseal plates was therefore relatively well preserved, but without any other evidence of viability.

The marrow exhibited loss of hematopoietic elements and fat spaces and replacement with fibrin strands. Occasional inflammatory cells were scattered throughout, as was eosinophilic cellular debris. In some specimens, marked inflammatory infiltrate was present with erosion of the epiphyseal plate (FIGURE 15; pg. 80).

All bone in the donor graft was necrotic. Osteocytes of the epiphyseal bone plate were either absent or pyknotic, as were those within the cortices and metaphyseal trabeculae. Reactive new bone was seen in between the trabeculae at the graft / host interface. In these regions, a marked osteoblastic response could be seen. This gave the appearance of a reparative healing process by the host, rather than one of active bone growth due to endochondral ossification.
FIGURE 15: Photomicrograph of non-viable epiphyseal plate transplant from non-immunosuppressed animal

Legend:
A - Empty osteocyte lacunae  
B - Absent epiphyseal capillary  
C - Weak staining of matrix  
D - Necrotic chondrocyte  
E - Inflammatory infiltrate, loss of osteoprogenitors
FIGURE 16: Serial photomicrographs from an unmanipulated epiphyseal plate showing appearance with WHO histological stain and immunohistochemistry for BrdU

Legend:
A - Proliferative-zone chondrocytes positive for BrdU uptake
FIGURE 17: Serial photomicrographs of transplanted epiphyseal plates with continuous immunosuppression (6 weeks) - with and without bone contact. Viability of both groups was confirmed by BrdU uptake.

Legend:

A - With bony fixation
A1 - WHO stain
A2 - BrdU immunohistochemistry

B - Without bony fixation
B1 - WHO stain
B2 - BrdU immunohistochemistry

All photomicrographs taken at 400 X magnification
3.2.2.4 Histological findings: immunosuppressed specimens

Immunosuppressed specimens had a markedly different appearance compared to the non-suppressed grafts. The histological features were very consistent with those of the normal, unmanipulated proximal tibial epiphyseal plates (FIGURE 16; pg. 81). Epiphyseal plate chondrocytes were basophilic with clear nuclear detail. Few empty lacunae were present in either the epiphyseal bone plate or the cartilaginous portion. The matrix was bright green in colour, indicating appropriate staining of matrix mucopolysaccharides (FIGURE 17; pg. 82).

The marrow retained a normal appearance in terms of hematopoietic tissue and fat spaces, with erythrocytes confined to capillaries and sinusoids. The osteocytes in both the cortex and trabeculae as well as the osteoblasts in the primary spongiosa were identical to those of the contralateral unoperated recipient tibia.

The most notable departures from normality seen in the immunosuppressed epiphyseal plate grafts related to the epiphyseal plate architecture. In some specimens, the epiphyseal plate demonstrated somewhat disorganized columnar orientation. Epiphyseal plate closure, seen initially on the radiographs, was visible in greater detail. The portion of the epiphyseal plate that underwent closure ranged from 40% to 70% (mean = 56.0% ± 13.4%). The open portion of the plate was always a single, contiguous segment with no intervening bony bridges.

The lucency of the metaphyseal area seen on the radiographs was due to absence or attenuation of most of the metaphyseal bone trabeculae. Osteopenia of this nature was not seen in the non-immunosuppressed specimens.
3.2.2.5 Fluorochrome labels

Although successive labels were visible in the metaphyseal area of the unmanipulated, control epiphyseal plates, several factors prevented satisfactory interpretation of the fluorochrome labels in the transplanted specimens. In the immunosuppressed specimens with bone contact, resorption of the metaphyseal trabeculae resulted in absence of much of the area in which the fluorochromes would have been deposited. Consistent with previous studies, non-immunosuppressed specimens failed to demonstrate any evidence of fluorochrome uptake. Because of the inability of the fluorochromes to contribute reliable adjunctive information, processing of the undecalcified sections was not performed for the remaining components of the investigation. Calculation of the rejection index outlined above (TABLE 1, pg. 30) was modified by elimination of the score for presence of the pre-sacrifice fluorochrome label.

3.2.2.6 Immunohistochemistry

Positive and negative controls confirmed that the immunohistochemical technique generated valid results without false positive or false negative findings. Histological sections of paraffin-embedded gut epithelium showed strong positive uptake of BrdU (not shown). Unmanipulated proximal tibial epiphyseal plates from each recipient rabbit demonstrated labeling of chondrocytes in the proliferative zone. These positive controls demonstrated that the BrdU compound had been properly administered, was present within the systemic circulation of all experimental animals, was incorporated by proliferating cells, and that the histological processing techniques preserved the BrdU epitopes. In rabbits that had not received BrdU, or when anti-BrdU antibody was withheld during immunohistochemical processing, labeling of epiphyseal plate chondrocytes was totally absent. These findings confirmed that the observed
staining of epiphyseal plate chondrocytes was solely due to the presence of BrdU within the tissue and not a false-positive artifact of the processing regimen itself.

The number of proliferative-zone cells positive for BrdU, expressed as percentage of all proliferative zone cells, was 14.7% ± 2.7% for the continuously immunosuppressed epiphyseal plates. This value was not significantly different from the unmanipulated control epiphyseal plates (13.2% ± 2.8%) but was in stark contrast to the non-immunosuppressed specimens, which showed no uptake of the BrdU label whatsoever.

**TABLE 4: Summarized Results of Preliminary Study B**
Continuous therapy x 6-weeks - immunosuppressed specimens vs. non-suppressed

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<thead>
<tr>
<th></th>
<th>IMMUNOSUPPRESSION</th>
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<tbody>
<tr>
<td></td>
<td>YES (n=5)</td>
<td>NO (n=5)</td>
</tr>
<tr>
<td>R</td>
<td>Gross Findings</td>
<td>Growth</td>
</tr>
<tr>
<td>E</td>
<td>Growth (mm)</td>
<td>5.25 ± 1.53</td>
</tr>
<tr>
<td>S</td>
<td>Bony Union (%)*</td>
<td>100</td>
</tr>
<tr>
<td>U</td>
<td>Pedicle Patency (%)*</td>
<td>100</td>
</tr>
<tr>
<td>L</td>
<td>Histology (% viable*)</td>
<td>100</td>
</tr>
<tr>
<td>T</td>
<td>BrdU Labeling Index (%)</td>
<td>14.7 ± 2.7</td>
</tr>
<tr>
<td>S</td>
<td>Plate Closure (%)</td>
<td>56.0 ± 13.4</td>
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</table>

* - percentage refers to the proportion of specimens demonstrating the parameter in question

3.2.2.7 *Ischemic controls*

The immunosuppressed specimens without microvascular anastomoses exhibited findings very similar to those without immunosuppression. Union of the graft with the iliac crest recipient site had occurred, but there was no evidence of increased longitudinal dimension by
gross, radiographic, or microscopic analysis. Histological specimens revealed diffuse necrosis of all cellular components of the graft. Epiphyseal plate lacunae were either empty or occupied by necrotic chondrocyte remains. Occasional mononuclear cells were visible in the metaphyseal region, which was otherwise replaced by an acellular, fibrinous infiltrate. The epiphyseal plate architecture was generally preserved, and did not show enlargement of the hypertrophic zone as did the non-immunosuppressed specimens. Immunohistochemical processing revealed no uptake of BrdU whatsoever.

Consolidated results from all investigations are presented at the end of this section (TABLE 9; pg. 94).

3.3 PRINCIPAL EXPERIMENT: Short-term immunosuppression

3.3.1.1 Gross Findings

All grafts in the principal experiment, including those with short-term immunosuppression and those without, had non-patent vascular pedicles at the time of sacrifice. The appearance of these vessel remnants was essentially the same as those of the non-immunosuppressed specimens from Preliminary study B. Other than the vascular pedicles, specimens that had received short-term immunosuppression appeared grossly similar to the continuously suppressed grafts from Preliminary study B; they had clearly increased in longitudinal dimension compared to their original postoperative size (TABLE 5; pg. 92).

3.3.1.2 Radiographic findings

Radiographic appearances of the specimen cross-sections were similar to those from Preliminary study B (FIGURE 18; pg. 88). The dimensions of the non-immunosuppressed specimens (°Fix°CsA, °Fix°CsA) were essentially unchanged from their original, post-operative
values (TABLE 5; pg. 92). The epiphyseal plates were open and metaphyseal trabecular density was similar to the unmanipulated proximal tibias. The short-term specimens with bony fixation ("Fix"CsA) shared the distinguishing features of the immunosuppressed grafts from Preliminary study B; increase in longitudinal dimension was evident, as was partial epiphyseal plate closure and metaphyseal osteopenia.

Specimens with short-term immunosuppression but without bone contact ("Fix"CsA) shared features of the two above groups. The grafts had grown in longitudinal dimension, but did not exhibit radiographic evidence of epiphyseal plate closure or metaphyseal osteopenia. Of the specimens with short-term immunosuppression, growth of the grafts without bone contact (10.28 mm ± 3.48 mm) was greater than those with bone contact (5.09 mm ± 1.85 mm) (TABLE 5; pg. 92). This difference was statistically significant (p(Bonferroni < 0.05).

3.3.1.3 Histological findings

The findings in the non-suppressed specimens, both with and without bone contact, were identical to those in the non-suppressed specimens from Preliminary study B. The epiphyseal plates demonstrated necrotic chondrocytes, absence of mucopolysaccharide staining, and occasional erosion by inflammatory infiltrates. These grafts were clearly non-viable, with the typical features of rejection previously described.

The appearance of specimens with short-term immunosuppression depended largely on the presence or absence of bony contact with the host; epiphyseal plates from specimens with bone contact were relatively normal whereas those without fixation were significantly compromised (FIGURE 19; pg. 89).
FIGURE 18: Radiographs of specimens from each experimental group in the main study. Growth occurred in those with immunosuppression. Metaphyseal osteopenia was evident only in the grafts with both immunosuppression and bone contact.

<table>
<thead>
<tr>
<th>Immunosuppression</th>
<th>Bone Contact</th>
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<tbody>
<tr>
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<tr>
<td>YES</td>
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<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>NO</td>
<td>NO</td>
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</tbody>
</table>

Legend:
Brackets denote margins of epiphyseal plate graft

= 5 mm
FIGURE 19: Serial photomicrographs of transplanted epiphyseal plates with short-term immunosuppression (6 weeks with, 4 weeks without), with and without bone contact. Only grafts with bone contact were viable, and were positive for BrdU detection by immunohistochemistry.

Legend:

A - With bone contact      A1 - WHO stain
A2 - BrdU immunohistochemistry
B - Without bone contact   B1 - WHO stain
B2 - BrdU immunohistochemistry

All photomicrographs taken at 400 X magnification
The epiphyseal plates from the specimens with bone contact (\textsuperscript{\textsuperscript{+}}\textsuperscript{Fix+CsA}) were virtually indistinguishable from those with 6-weeks of continuous immunosuppression in Preliminary study B (FIGURE 17., pg. 82). Partial epiphyseal plate closure had likewise occurred in this experimental group only (TABLE 8.; pg. 93). Some differences were visible in the metaphyseal area, however. These included some degeneration and loss of osteoprogenitors, increased fibrin deposition and cellular debris. Metaphyseal capillaries were somewhat indistinct, but had not progressed to the stage of destruction and red blood cell extravasation. The overall impression from these histological specimens was that of viable epiphyseal plates with some early rejection in the metaphyseal area.

Two specimens from the short-term group with fixation (\textsuperscript{\textsuperscript{+}}\textsuperscript{Fix+CsA}) had anomalous findings. Unlike the other specimens in this group, the epiphyseal plates in these grafts were non-viable and demonstrated marked degeneration of the cellular components. Review of the animal records revealed that these two specimens came from animals that had developed Cyclosporine toxicity that necessitated reduction of the dose to 2.5 mg/kg/day for a period of 5 days. These specimens were deemed non-representative of the group and were therefore removed from the analysis.

Specimens without bone contact (\textsuperscript{\textsuperscript{+}}\textsuperscript{Fix+CsA}) showed little histological evidence of preserved viability. Scant difference existed between the appearance of these specimens and the non-immunosuppressed grafts from Preliminary study B. In several specimens, the cartilaginous matrix demonstrated relatively strong uptake of the WHO stain although the epiphyseal plate chondrocytes were necrotic. The rejection index correlated with the overall histological appearances of the specimens. The mean index for the specimens without bone contact was 7.4
± 1.6 – a value characterized as ‘intense rejection’ according to the classification previously outlined (TABLE 1; pg. 30).

### 3.3.1.4 Immunohistochemistry

The results of the immunohistochemistry confirmed the impressions gained from the histological analysis. Consistent with their non-viable appearance, epiphyseal plates from grafts without short-term immunosuppression both with (\(^{8}\)Fix\(^{9}\)CsA) and without bone contact (\(^{9}\)Fix\(^{9}\)CsA) were negative for BrdU uptake. Similarly, no uptake was discernible in the short-term specimens without bone contact (\(^{9}\)Fix\(^{9}\)CsA) (TABLE 7; pg. 93). The short-term specimens with bone contact (\(^{9}\)Fix\(^{9}\)CsA), however, exhibited strong uptake of the BrdU label (FIGURE 19; pg. 89). The labeling index for these specimens was 16.0% ± 2.9%. Although the experimental model does not permit statistical comparison with control epiphyseal plates from the unmanipulated tibias, the values were close in actual magnitude and standard deviation (16.0% ± 2.9% for \(^{9}\)Fix\(^{9}\)CsA versus 14.1% ± 2.2% for unmanipulated).

### 3.4 MORBIDITY AND MORTALITY

The total number of recipient rabbits, including those used in preliminary studies, was 71. Three of the recipient rabbits died prematurely; one died from an anesthetic complication prior to transplantation, one from a spinal fracture, and one from severe wasting secondary to Cyclosporine toxicity\(^{107}\). Two additional rabbits suffered moderate wasting characteristic of Cyclosporine toxicity, but this resolved following reduction of the dose to 2.5 mg/kg/day for a period of 5 days.

Two rabbits developed superficial wound infections that were successfully treated with antibiotics.
### TABLE 5: Results of principal experiment - Growth

<table>
<thead>
<tr>
<th>Growth (mm)</th>
<th>SHORT-TERM IMMUNOSUPPRESSION</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BONE CONTACT</td>
<td>YES</td>
<td>5.09 ± 1.85&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.060 ± 0.074&lt;sup&gt;b,l&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>10.28 ± 3.48&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.096 ± 0.049&lt;sup&gt;c,l&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2-way ANOVA: $F_{\text{immuno}} = 169.379 \ (p < 0.001); \ F_{\text{contact}} = 19.923 \ (p < 0.001)$

Multiple pairwise comparisons of group means (Bonferroni):

- Pairs with common letter are significantly different ($p < 0.05$)
- Pairs with common numeral are not significantly different

### TABLE 6: Results of principal experiment - Rejection index

<table>
<thead>
<tr>
<th>Rejection Index</th>
<th>SHORT-TERM IMMUNOSUPPRESSION</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BONE CONTACT</td>
<td>YES</td>
<td>14.6 ± 1.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.8 ± 0.07&lt;sup&gt;b,l&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>7.4 ± 1.6&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>5.6 ± 0.7&lt;sup&gt;c,l&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2-way ANOVA: $F_{\text{immuno}} = 219.195 \ (p < 0.001); \ F_{\text{contact}} = 103.751 \ (p < 0.001)$

Multiple pairwise comparison of group means (Bonferroni):

- Pairs with common letter are significantly different ($p < 0.05$)
- Pairs with common numeral are not significantly different
### TABLE 7: Results of principal experiment – BrdU labeling index

<table>
<thead>
<tr>
<th>BrdU index (%)</th>
<th>SHORT-TERM IMMUNOSUPPRESSION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td><strong>BONE CONTACT</strong></td>
<td>YES</td>
<td>16.0 ± 2.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>0.0 ± 0.0&lt;sup&gt;a,1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2-way ANOVA: \( F_{\text{immuno}} = 409.404 \) (\( p < 0.001 \)); \( F_{\text{contact}} = 409.404 \) (\( p < 0.001 \))

Multiple pairwise comparison of group means (Bonferroni):

- \( a,b,c \): Pairs with common letter are significantly different (\( p < 0.05 \))
- \( 1,2,3 \): Pairs with common numeral are not significantly different

### TABLE 8: Results of principal experiment – Epiphyseal plate closure

<table>
<thead>
<tr>
<th>Closure (% closed)</th>
<th>SHORT-TERM IMMUNOSUPPRESSION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td><strong>BONE CONTACT</strong></td>
<td>YES</td>
<td>34.5 ± 11.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td>0.0 ± 0.0&lt;sup&gt;a,1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2-way ANOVA: \( F_{\text{immuno}} = 111.195 \) (\( p < 0.001 \)); \( F_{\text{contact}} = 111.195 \) (\( p < 0.001 \))

Multiple pairwise comparison of group means (Bonferroni):

- \( a,b,c \): Pairs with common letter are significantly different (\( p < 0.05 \))
- \( 1,2,3 \): Pairs with common numeral are not significantly different
### TABLE 9: Consolidated summary of results from all phases of investigation process

<table>
<thead>
<tr>
<th>INVESTIGATION PHASE</th>
<th>PURPOSE</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MODEL DEVELOPMENT</td>
<td>Establish model</td>
<td></td>
</tr>
<tr>
<td>Angiography</td>
<td>Define vascular anatomy of</td>
<td>Epiphyseal plate vascular supply amenable to surgical isolation</td>
</tr>
<tr>
<td>Corrosion casts</td>
<td>proximal tibial epiphyseal plate</td>
<td></td>
</tr>
<tr>
<td>Surgical dissections</td>
<td>Develop a surgical procedure</td>
<td>Successful development of surgically reproducible microvascular</td>
</tr>
<tr>
<td></td>
<td>for microvascular transplantation of</td>
<td>transplantation procedure</td>
</tr>
<tr>
<td></td>
<td>proximal tibial epiphyseal plate</td>
<td></td>
</tr>
<tr>
<td>Radioactive microsphere injections</td>
<td>Quantify perfusion to epiphyseal plate graft in unmanipulated and transplanted states</td>
<td>No significant difference in graft perfusion between transplanted and unmanipulated specimens</td>
</tr>
<tr>
<td>India ink injections</td>
<td>Evaluate distribution of bloodflow in epiphyseal plate grafts, before and after transplantation</td>
<td>Perfusion to both epiphyseal and metaphyseal regions of graft preserved in all cases</td>
</tr>
<tr>
<td>2. PRELIMINARY STUDIES</td>
<td>Model validation</td>
<td></td>
</tr>
<tr>
<td>A. Bone union</td>
<td>A. Evaluate time to bone union</td>
<td>Bone union complete in all grafts within 4-weeks post-surgery</td>
</tr>
<tr>
<td>B. Continuous immuno-suppression</td>
<td>B. Evaluate epiphyseal plate viability with continuous immuno-suppression, at 6 and 10 weeks post-transplantation, with and without bone contact</td>
<td>All epiphyseal plates with continuous immunosuppression were viable at all time points, both with bone contact and without. Grafts without immunosuppression were non-viable</td>
</tr>
<tr>
<td></td>
<td>- 6 weeks duration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 10 weeks duration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- with bone contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- without bone contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- ischemic controls</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Evaluate viability with immuno-suppression but without micro-surgical revascularization</td>
<td>All ischemic grafts were nonviable</td>
</tr>
<tr>
<td>3. PRINCIPAL EXPERIMENT</td>
<td>Efficacy of Short-term immunosuppression</td>
<td></td>
</tr>
<tr>
<td>Short-term immuno-suppression</td>
<td>Evaluate viability of epiphyseal plate transplants with short-term immunosuppression, both with and without bone contact</td>
<td>Epiphyseal plates with short-term immunosuppression were viable only in presence of bone contact; all others were non-viable</td>
</tr>
<tr>
<td>A. With bone contact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Without bone contact</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter Four:

4 DISCUSSION
4.1 ACCEPTANCE OF MAIN HYPOTHESIS

Vascularized allograft epiphyseal transplants with bone contact were viable four weeks following withdrawal of short-term immunosuppression. Despite an identical immunosuppression regimen, transplants without bone contact were non-viable, and showed histological features consistent with advanced rejection. These findings provided the evidence necessary to accept the main hypothesis of this experiment – specifically, that an allograft vascularized epiphyseal plate transplant with minimal adjacent bone will remain viable after withdrawal of short-term immunosuppression, provided bone contact is maintained.

The experimental aims of this investigation differed from that of previous studies of epiphyseal plate transplantation. Most of these prior studies emphasized the measurement of growth after transplantation, whereas this investigation focussed on epiphyseal plate viability. As such, this investigation is the first to use immunohistochemical markers of cellular proliferation in the context of epiphyseal plate transplantation. The binary nature of the outcome strengthens the implications of the results. Had epiphyseal plates been viable in transplants both with and without fixation, we would be unable to speculate on the possible role of bone contact, and hence neovascularization, in the survival of these grafts following short-term immunosuppression.

These results have encouraging implications for the eventual clinical application of vascularized allograft epiphyseal plate transplants. They are the first to conclusively show that alterations in graft design have impact on survival following withdrawal of short term immunosuppression.
4.2 CHOICE OF ANIMAL MODEL

Allograft vascularized epiphyseal plate transplants have been performed in dogs, rats, and rabbits using a variety of donor and recipient sites\textsuperscript{32}. Each of these models has individual strengths and weaknesses, as does the one used in the current study. The rationale for the use of a rabbit model, and the particular donor and recipient site was based on several considerations.

Rabbits have been used successfully at our institution for previous investigations of vascularized epiphyseal transplants\textsuperscript{33, 88, 203}. We are familiar with their handling, housing needs, pre, peri and postoperative surgical management and the financial requirements associated with their care. Anatomical and histological features of the rabbit musculoskeletal system are also well known to us. In addition, the size of the rabbit was favorable for the microvascular transfer of the proximal tibial growth plate. Together, these features made the use of rabbits attractive for the current study.

The disadvantages of a rabbit model are largely related to immunological issues. When performing investigations on the behavior of allograft transplants, knowledge of the genetic makeup of the animals is important to ensure that donor and recipient are indeed allogeneic. Early studies on allograft vascularized bone and epiphyseal plate transplants, however, were performed in outbred animal models which made certification of allogenicity difficult or impossible to obtain. For this reason, investigators encouraged the use of inbred animal strains for further experiments\textsuperscript{237}. The use of inbred strains is appealing because allogenicity of the donor and recipient can be assured without the need for MHC haplotyping of the individual animals. Because all animals of a given strain are genetically identical, all grafts can be transplanted across a consistent histocompatibility barrier. This feature reduces the potential for variability in the aggressiveness of the rejection response. In addition, inbred strains permit the
use of syngeneic isografts to evaluate the effects of the surgical procedure itself without the responses of the immune system as a variable.

Most recent investigations of allograft vascularized bone and cartilage transplants have used rat models\textsuperscript{109, 131, 138, 180}. Because commonly used rat strains are commercially available and well characterized genetically, immunological reagents such as monoclonal antibodies have been developed to react with tissue components of specific strains thereby increasing the sophistication of analytical techniques available to the researcher.

In contrast, only outbred rabbits are currently available from commercial sources. Establishing and maintaining inbred rabbit strains is difficult because rabbits tend to lose viability as the coefficient of inbreeding of a colony rises\textsuperscript{50}. In the absence of MHC haplotyping, it is difficult to ascertain the extent of antigenic disparity between two rabbits, whether both are from the same or different colonies.

Attempts were made early in the course of this study to modify this project for execution in a rat model, but technical difficulties prevented success. The iliac crest of the rat was too narrow and thin to serve as a recipient site, and no other suitable sites existed. Key features of the graft design used in this study were inclusion of a minimal amount of bone on both sides of the epiphyseal plate, and the ability to implant it in a bony site. Rabbits proved to be the smallest laboratory animal in which this procedure was technically possible.

To overcome the weaknesses associated with the use of outbred rabbits, extensive MHC haplotyping of donors and recipients was performed in a previous study at our facility. Using RFLP analysis of the rabbit MHC it was determined that, in a randomly chosen, non-sibling, pair of rabbits from our supplier, the likelihood of syngeneity was less than 0.1 percentage\textsuperscript{34}. The breeding practices of our supplier have not changed since that study was conducted (Personal
communication, June 1996, Charles River Laboratories). Because of the chance of obtaining a syngeneic donor/recipient pair was so small, we felt that molecular haplotyping of the animals, a time-consuming and costly process, was unwarranted for the current study.

The results of the preliminary studies were typical of those seen following transplantation of vascularized epiphyseal plates across a strong histocompatibility barrier. At less-than 14 days post-transplantation, epiphyseal plate grafts in animals without immunosuppression were non-viable, showing no uptake of BrdU in proliferative zone chondrocytes. The pedicles in each case were non-patent and the histological specimens showed evidence of acute rejection as defined by published criteria^{92, 94}. All non-immunosuppressed controls performed in the principal experiment had similar findings. Rejection of vascularized bone grafts performed across weak histocompatibility barriers has a much slower evolution, and results in a demonstrably different histological appearance in the early post-operative period compared to those performed across a strong barrier^{94}. Thus, after withdrawal of short-term immunosuppression, the improved viability of the fixed compared to the non-fixed grafts cannot be easily attributed to undetected genetic histocompatibility between donors and recipients.

4.2.1 Validation of the Model

4.2.1.1 Donor epiphyseal plate graft

The whole-knee transplant model has been used to investigate aspects of allograft vascularized epiphyseal plate transplantation, both in rabbits^{33, 75, 240} and in rats^{63, 92, 167, 168, 236}. These studies confirmed that the knee, including the proximal tibial and distal femoral epiphyseal plates, was adequately vascularized when isolated on the popliteal arteriovenous pedicle. The graft design used in this study was based on the whole-knee model but incorporated
several important differences. In order to transplant the proximal tibial epiphyseal plate alone, the knee was disarticulated and the entire distal femur component was removed. In addition, the articular cartilage of the proximal tibia was removed and the graft included much less adjacent bone from the tibial diaphysis. Because of these modifications, steps were taken to affirm that the vascularity and viability of the epiphyseal plate were preserved with the new graft design (TABLE 9: pg. 94).

Multiple anatomical assessments clearly showed that the epiphyseal and periosteal vessels issued from the popliteals in the immediate vicinity of the epiphyseal plate. In this manner, they were amenable to preservation even with the small size of transplant proposed in this study. Qualitative and quantitative methods for assessment of perfusion revealed that the removal process did not significantly alter the graft vascularity.

The possibility existed, however, that the distribution of bloodflow within the epiphyseal plate graft was altered as a result of the surgical dissection. This was investigated by injection of India ink and barium sulphate into the vascular pedicle of several specimens. In each case histologic (India ink) and radiographic (barium sulphate) analysis confirmed the preservation of vascular supply to the epiphyseal and metaphyseal regions of the epiphyseal plate graft.

The next step in the validation of the model was to demonstrate that epiphyseal plate viability was indeed maintained following transplantation. Preliminary study B was undertaken to establish this fact, and showed that all outcome measures - gross, radiographic, histological and immunohistochemical - were compatible with preserved graft viability following 6 weeks of continuous immunosuppression. These findings were in stark contrast to the non-immunosuppressed transplants, where evidence of viability was uniformly absent. With this
data, the model was deemed valid and therefore suitable to confirm or refute the main experimental hypothesis.

4.2.1.2 Recipient Site

The iliac crest recipient site provided non-weightbearing, well-vascularized cancellous bone and the opportunity for bony union on both epiphyseal and metaphyseal sides of the epiphyseal plate. The preliminary studies confirmed the suitability of this site; rapid bony union occurred between the iliac bone and the epiphyseal plate grafts in all cases. The extent of host neovascularization of the epiphyseal plate graft was not specifically quantified. Indirect evidence of this process was provided by injection of India ink into the vascular pedicle of the graft following 6 weeks of continuous immunosuppression. The bone of the ilium (recipient) was blackened in addition to that of the epiphyseal plate graft, confirming that vascular communication had indeed been established between the graft and the recipient site bone within the period of immunosuppression.

4.3 OUTCOMES - EVALUATION OF EPIPHYSEAL PLATE VIABILITY

Assessments of epiphyseal plate viability are the major outcomes in this study. In previous experiments, viability of a transplanted epiphyseal plate was generally confirmed by evaluation of function (growth) and histological features\textsuperscript{33, 236, 237, 240}. The emphasis on function is understandable given that the goal of an epiphyseal plate transplant, from a reconstructive standpoint, is continued longitudinal growth. Although growth assessments were performed in this study, the unique characteristics of the current model rendered them relatively less valuable. Of the available techniques to evaluate epiphyseal plate viability - morphometric, histological, and immunohistochemical, the latter were the most definitive.
4.3.1 Morphometric techniques to assess epiphyseal plate viability

4.3.1.1 Radiographic techniques

Evidence of epiphyseal plate growth after withdrawal of immunosuppression would constitute proof of sustained viability. Data of this nature requires the ability to measure the temporal growth pattern of the graft. This has been performed in previous studies using serial measurement of distance between radiographic landmarks, and attributing the change to epiphyseal plate growth. The same technique was used in this experiment and consisted of a metal screw in the tibial diaphysis to quantify growth of the control (unoperated) proximal tibial epiphyseal plate. Growth of the transplanted epiphyseal plate, however, was more difficult to assess. Although the iliac crest recipient site served the purposes of this study, it did present several disadvantages. In-situ radiographic imaging of the graft was complicated by the presence of superimposed skeletal structures. Other imaging modalities (CT, MRI) were impractical. Serial radiographic measurements of graft size were therefore impossible, and calculation of growth could be made only after removal of the specimens. Consequently, the growth data for the epiphyseal plate transplants cannot be clearly attributed to any specific period following transplantation.

4.3.1.2 Histomorphometric techniques

Multiple fluorochrome labels facilitate measurement of the amount of bone growth in the time interval between the administration of the different labels. Attempts were made to use this strategy to calculate growth occurring after withdrawal of immunosuppression. The first fluorochrome, oxytetracycline, was given 24 hours post-transplant to document successful revascularization. The second, DCAF, was given at the time of withdrawal of
immunosuppression. The third and final fluorochrome, xylenol orange, was administered immediately before sacrifice. In this manner, measurement of the mineral apposition rate between the second (DCAF) and the third (xylenol orange) fluorochrome labels would have represented growth that had occurred in the period without immunosuppression.

Unfortunately, an unexpected phenomenon prevented the interpretation of the fluorochromes in our specimens. As is discussed further below, the bone in the metaphyseal area of the fixed specimens underwent significant resorption. Essentially, the bony trabeculae in which the initial fluorochromes would have been deposited were absent, and therefore unavailable for analysis. Due to their significant weaknesses, growth comparisons between groups were not emphasized in this investigation. In the absence of serial fluorochrome labels, histomorphometric assessments of epiphyseal plate growth are a weak indicator of viability in this model.

4.3.2 Histological techniques to assess epiphyseal plate viability

Evaluation of the histological features of the epiphyseal plate was one of the methods used to assess viability. Although descriptive histology does provide some indication of epiphyseal plate viability, it does not readily lend itself to quantification. Some features were quantified, however, according to the guidelines of the previously reported rejection index\textsuperscript{94}.

Results of the rejection index did correlate with the actual viability. Those with low rejection indices had little growth, non-viable general histological appearance, and absence of BrdU uptake. Weaknesses associated with this method stem from the fact that it is only semi-quantitative and involves a certain amount of subjectivity in the assignment of scores. The grading system assigns equal weight to all criteria, whereas in reality some parameters may be more important indicators of viability than others may.
The lack of the fluorochrome labels required modification of the index for the purposes of this study. The reliability of this modified form of the index has not been specifically established, nor did it offer adjunctive information with respect to epiphyseal plate viability. For this reason, no attempt was made to perform statistical comparison of the rejection indices between the various experimental groups.

The histological specimens did provide important information regarding the general biological processes occurring in the epiphyseal plate transplants. In addition, they facilitated assessment of the extent of bony union between grafts and the recipient site. However, they did not provide information sufficient to confirm or refute viability in all cases.

4.3.3 Immunohistochemical techniques to assess epiphyseal plate viability

Detection of BrdU-labeled cells was performed with peroxidase-conjugated anti-BrdU antibody, a technique previously proven valid for decalcified, paraffin-embedded sections of epiphyseal plates. Appropriate positive and negative controls confirmed these conclusions in our model. Results of this technique were consistent with those of other studies in terms of distribution of the labeled cells and labeling index. Although quantitative labeling indices were obtained, statistical comparison of values between groups was not necessary because of the binary outcomes obtained; in the various experimental groups, BrdU labeling was either present or absent. This finding made assessment of epiphyseal plate viability straightforward.

4.4 EXPERIMENTAL TIME LINE

The ideal duration of immunosuppression would be the shortest period compatible with bony union and adequate neovascularization of the graft. At the beginning of this project, these
approximate time points were unknown. Preliminary study A was undertaken to evaluate the
time of bony union of the graft with the recipient site, using varied follow-up times from 10 days
through 6 weeks postoperatively. Although our data demonstrated that bony union was complete
well before withdrawal of immunosuppression, difficulties including non-existence of
appropriate molecular probes prevented direct quantification of the extent of graft
neovascularization. Intravital microscopic analysis of healing bone grafts in the rabbit quantified
the rate of blood vessel ingrowth at approximately 0.2 – 0.4 mm/day. Given the small
dimensions of the graft used in this project, the immunosuppression interval was likely of
sufficient duration for host blood vessels to reach the proximity of the transplanted epiphyseal
plate.

In the principal experiment, four weeks elapsed following withdrawal of immunosuppres-
sion. An interval of sufficient length was required such that features of diminished growth plate
viability would manifest to an extent detectable by our evaluation techniques. The preliminary
studies established that transplanted epiphyseal plates were clearly nonviable within 10 days in
the absence of immunosuppression. These findings were further accentuated in the control, non-
immunosuppressed specimens from the principal experiment and confirmed that the withdrawal
period was of sufficient duration.

4.5 IMMUNOCOMPETENCE

States of allograft unresponsiveness or tolerance have occasionally been reported following
withdrawal of Cyclosporine therapy in rabbits<sup>97</sup>. In other models of allograft organ
transplantation, restoration of immunocompetence following withdrawal of immunosuppression
has been evaluated with donor-strain second set tissue grafts<sup>145, 210</sup> and <i>in vitro</i> cell-mediated
lymphocytotoxicity assays\textsuperscript{131}. Neither of these methods was practical in our model. Although direct confirmation of immunocompetence was not performed, the control groups did provide evidence to support the recipients' ability to reject tissue of donor origin. Short-term immunosuppression was administered to grafts both with and without bone contact. With respect to immunocompetence, the status of the recipients in both groups would have been similar following withdrawal of the Cyclosporine. In spite of this fact, the findings in the two groups were markedly different. The non-fixed specimens showed complete loss of viability and typical features of advanced rejection, whereas grafts with skeletal fixation were viable. Further support for the immunocompetence of these animals was provided by examination of the vascular pedicles at the time of sacrifice. In each case, the vessels were non-patent, fibrous strands. Following withdrawal of immunosuppression, had the animals lacked the ability to reject tissue of donor origin we would have expected most, if not all of them to remain patent (as they had in the continuously immunosuppressed animals).

4.6 EXPERIMENTAL DESIGN

4.6.1 Controls

In general, the ideal control for a musculoskeletal allograft is an autograft of a similar bone segment implanted through an identical surgical approach and with the same fixation technique\textsuperscript{205}. An autograft was not possible in this model; surgical isolation of the graft required extensive dissection about the knee, division of all major muscle groups, knee ligaments and the patellar tendon. The operative procedure destabilized and devascularized the remaining portion of the lower limb. If an autograft were performed, the animal would have been left without a viable lower extremity. Sham operations were not possible for the same reasons. Theoretically,
amputation of the dissected donor limb would have permitted survival of the animal following an autograft transplant, but such actions were incompatible with ethical treatment of the animals and the guidelines of the Animal Care Committee at our institution.

Although ideal controls were not possible in this investigation, their absence does not alter our ability to confirm the main hypothesis. The variables controlled for by the execution of autografts, syngeneic isografts, or sham operations would not have affected epiphyseal plate viability as determined by immunohistochemical techniques.

4.6.2 Randomization

Randomization of subjects to various experimental groups reduces systematic bias in a study and increases its internal validity\(^1\). The break in full randomization of the principal experiment therefore deserves consideration.

The principal experiment was randomized in two segments - animals without immunosuppression and those that received short-term immunosuppression (APPENDIX C: 120). Because this study made use of laboratory animals from a single supplier, all of the same age, the equivalence of experimental groups would have been high. Because differences in the animal subjects themselves were unlikely to be a factor, the break in full randomization would be of significance if systematic differences existed in the experimental protocol used in these two segments. The operative procedure, processing, and analytical techniques were conducted in an identical manner for all groups. However, the animals without short-term immunosuppression received surgery at an earlier stage of the experiment than did those with immunosuppression. Theoretically, animals receiving surgery later in the course of the experiment may have enjoyed greater success due to a position higher in the ‘learning curve’ of the surgeon. In reality, however, many transplants had been performed by the surgeon prior to beginning the principal
experiment, both in the context of preliminary studies and in adjunctive investigations. In addition, the consistent lack of viability of the non-suppressed epiphyseal plates, compared to those with immunosuppression, suggests that technical variances had little influence on the observed results.

In the principal experiment, the groups of most importance to the conclusions of this study were those receiving short-term immunosuppression either with or without bone contact. Animals were randomized to these two groups, thereby minimizing bias due to unidentified variables or other factors.

4.7 EPIPHYSEAL PLATE FUNCTION

Although evaluation of epiphyseal plate function was not the specific focus of this investigation, certain findings in the transplanted epiphyseal plates were compelling and worthy of discussion. Most notable was the presence of metaphyseal osteopenia and epiphyseal plate closure in the immunosuppressed (short-term and continuous) grafts with bone contact. The necessary conditions for the development of these features must therefore have been, A) Microsurgical revascularization of the graft with immunosuppression and, B) Location within the iliac crest recipient site (bone contact).

4.7.1 Metaphyseal osteopenia

Resorption of metaphyseal trabeculae has been noted by other authors following heterotopic transplantation of rat knee allografts to non-weightbearing positions\textsuperscript{168}. This finding was attributed to predominance of osteoclastic activity over osteoblastic, but occurred to a much lesser extent than that seen in our specimens. In the unloaded, heterotopic sites, the grafts lacked the physiologic stimulus for new bone formation or remodeling resulting from strain\textsuperscript{158}. 
Nonetheless, only the grafts with bone contact showed significant osteopenia. If absence of weightbearing was the only feature contributing to the metaphyseal osteopenia, then it should have occurred equally in grafts both with and without bone contact.

The unfixed group lacked the opportunity for revascularization from the host. Intravital microscopic analysis of bone graft healing in rabbits demonstrated that bone resorption only occurred after full revascularization had taken place. In poorly vascularized grafts, bone remained structurally unchanged for several months. This finding was consistent with those from the epiphyseal plate grafts transplanted to the iliac crest but without immunosuppression. Similarly, grafts with immunosuppression but without microsurgical revascularization (ischemic controls) exhibited preserved, albeit necrotic, bony architecture.

In the current study, it did not appear that bone contact alone was sufficient for development of metaphyseal osteopenia. The resorption of trabeculae was most consistent with a synergistic effect of successful microsurgical revascularization combined with bone contact.

Delineation of the true mechanism involved would require multiple adjunctive tests. Metaphyseal osteopenia was an unexpected observation evaluated qualitatively in the specimens. This phenomenon would have been approached differently were it a prime objective of the study. Assessment of the degree of resorption would require specific analysis by quantitative histomorphometry, however, and is beyond the focus of this investigation.

4.7.2 Epiphyseal plate closure

Certain of the findings in our transplanted specimens were consistent with previously reported effects of diminished weightbearing on vascularized epiphyseal plate grafts. Following heterotopic transplantation to a non-weightbearing recipient site, epiphyseal plates showed loss of chondrocyte columnar arrangement and segmental areas of epiphyseal closure with bony
bridge formation\textsuperscript{63, 93}. Although published photographs of this phenomenon in rat knee transplants\textsuperscript{180} were similar to our own, the absence of closure in the grafts without bone contact renders diminished weightbearing unlikely as the sole causal factor. In addition, the areas of closure seen in the rat studies\textsuperscript{63, 93, 180} were multiple and dispersed throughout the epiphyseal plate – unlike ours which consisted of a single, contiguous segment of closure in affected plates.

Partial epiphyseal plate closure has been reported following insults of various types including trauma\textsuperscript{159}, internal fixation\textsuperscript{31}, ischemia\textsuperscript{201}, and neural, vascular and metabolic abnormalities\textsuperscript{169, 215}. Factors of this sort, however, would have been shared by all transplants with short-term immunosuppression, and cannot be implicated in the partial closure seen in the grafts with bone contact.

Physiologic closure of epiphyseal plates occurs at skeletal maturity, although at different times in different epiphyses. In New Zealand White rabbits, closure of the proximal tibial epiphyseal plate, as determined by histological assessment, occurred from 25-32 weeks of age (mean 28 weeks)\textsuperscript{122}. In the current experiment, rabbits were approximately 20 weeks of age at the time of sacrifice and therefore unlikely to experience plate closure due to physiologic aging alone. In addition, the unmanipulated control proximal tibial plates, also of the same age, showed no evidence of closure.

Normal limbs experience dynamic loading forces due to effects of muscular activity, body weight, and accelerations and decelerations of the superimposed body mass\textsuperscript{51, 154}. These forces, in turn, affect the growth response of epiphyseal plates\textsuperscript{82}. In our model, isolation of the graft within a bony site resulted in a departure from the natural biomechanical environment of the epiphyseal plate.
Although the epiphyseal plates with bone contact were not subject to weight-bearing and related forces, they would have experienced loading due to growth within a site of essentially fixed dimensions. Inspection of the measured growth in the principal experiment suggests that the iliac crest recipient site provided a restrictive effect. Growth of the grafts without bone contact was 10.28mm ± 3.48mm, whereas those with bone contact grew 5.09mm ± 1.85mm. The fact that observed growth in the grafts without bone contact was almost two-fold greater than those with bone contact poses an interesting paradox, given that those without contact were non-viable at the time of sacrifice. The relative “excess” growth in grafts without bone contact must have occurred prior to their loss of viability, likely within the immunosuppression interval. The grafts with bone contact were viable for a longer period than those without, but were limited in either their rate and/or total extent of growth. It is likely that growth of the epiphyseal plate graft within the bony recipient site resulted in static compressive forces generated against the epiphyseal plate relatively early in the immunosuppression interval.

The effects of compressive forces on rabbit epiphyseal plates was investigated by the application of external fixation devices across femoral and tibial epiphyseal plates. Static compressive forces resulted in diminished longitudinal growth, which ceased entirely with sustained forces above 38N. In the plane of the pins, where the forces were highest, were noted the greatest effects on the epiphyseal plates. These included chondrocyte necrosis and epiphyseal plate closure with bony bridge formation. Interestingly, closure in our specimens was generally aligned with the areas of maximum bracketing of the epiphyseal plate graft by the iliac crest.
4.8 FUTURE DIRECTIONS

4.8.1 Further development of the current model

In this study, epiphyseal plate viability was demonstrated 4 weeks after withdrawal of immunosuppression. Perhaps rejection (and loss of epiphyseal plate viability) would have occurred if the grafts had been followed for a longer period after withdrawal of immunosuppression. Experimental designs similar to the one used in this experiment, but with longer follow-up periods, would be valuable to determine total length of time in which viability is maintained. Information of this sort would be vital prior to performing analogous epiphyseal plate transfers in humans.

The results of this experiment suggest that the opportunity for graft neovascularization is important for graft survival in the absence of immunosuppression. The specific biology and behavior of the donor and recipient microcirculation would therefore be an important topic for future study. After withdrawal of immunosuppression, the remaining tissues of donor origin would be subject to immunological attack, possibly making the neo-vessels vulnerable to non-specific tissue destruction secondary to the donor antigen specific response\(^{229}\). The small vessels may be responsive to the vasoactive and thrombogenic factors released during interactions of sensitized T cells and their targets\(^{62}\). Consequently, the duration for which the neo-vessels would be able to support the graft is uncertain. The ability to histologically discriminate vessels of donor and recipient origin would be difficult in outbred animals such as rabbits. Potentially, transplantation of epiphyseal plates from male into female rabbits would permit analysis of the neovascularization process and its temporal changes after transplantation. Male tissue could be identified by \textit{in-situ} hybridization of a probe to the testis-determining gene located on the
mammalian Y chromosome (Dr. E. Eicher, Jackson Laboratories, 1996, Personal communication).

Immunocompetent cells are contained within the marrow of epiphyseal plate transplants. Theoretically, graft-versus-host reactions could result when grafts of this type are transplanted into an immunosuppressed recipient. The extent to which these responses occur would be important to establish prior clinical application in humans.

4.8.2 The role of new animal models

The ultimate goal of allograft epiphyseal plate transplantation is reliable, continued longitudinal growth. Given that preservation of epiphyseal plate viability is a realistic possibility with short-term immunosuppression, investigation of the dynamics of epiphyseal plate function (growth) would be a logical and necessary focus of future studies.

Epiphyseal plates worthy of reconstruction by allograft vascularized transplantation would be those of the extremity long bones. These bones are largely cortical in nature and are subject to repetitive biomechanical loads. As discussed in the introduction, cortical and cancellous bone grafts undergo somewhat different processes of healing and neovascularization. Thus, epiphyseal plate transplantation to a recipient site in a long bone, as opposed to the iliac crest, may have important implications on survival of the allograft following withdrawal of immunosuppression. In addition, the role of biomechanical forces on vascularized epiphyseal plate transfers has not yet been adequately addressed. New animal models, with a different set of well-defined outcome measures, will be required to satisfactorily investigate these issues.
4.8.3 Emerging trends in transplantation

New immunosuppressive agents such as rapamycin\textsuperscript{121}, mycophenolate mofetil\textsuperscript{142, 227}, leflunomide\textsuperscript{16}, and brequinar sodium\textsuperscript{55, 120} offer the ability to target specific arms of the immune response. In this regard, they hold promise for suppression of host-anti-graft responses with a lower therapeutic index than current drugs and an improved side-effect profile. The roles of these new medications are under evaluation in clinical trials. Although side effects may be reduced with new immunosuppressants, in the absence of other strategies they will likely require long-term administration. In addition, the risks associated with immune-compromise will likely continue to hinder the use of chronic immunosuppression for non life-threatening applications.

An emerging field may offer the ability to transplant functional tissues without either donor site morbidity or the risk of rejection. Tissue engineering is the application of the principles and methods of engineering and the life sciences towards development of biological substitutes to restore, maintain or improve function\textsuperscript{148}. Much interest has developed in the use of biomimetic synthetic polymers, patterned in three-dimensions, to generate model multicellular tissue architectures. These synthetic extracellular matrices, some biocompatible and others biodegradable, when seeded with autologous cells \textit{in vitro}, provide a temporary scaffolding to guide new tissue growth and organization\textsuperscript{126}.

Promising results have been obtained in the creation of viable constructs from a variety of tissue types. Cartilage structures with specific three-dimensional shapes and cellular organizations have been formed by incorporating cultured chondrocytes into various polymer substrates\textsuperscript{39, 40, 77, 101, 189, 192, 193}. Bone formation has been achieved in a similar manner with the use of osteoblasts\textsuperscript{175, 225, 226}. Polymeric tubes seeded with endothelial cells \textit{in vitro} may allow construction of neo-vessels\textsuperscript{139, 140, 153, 233} with specific antigenic characteristics.
Although certain individual component tissues of vascularized epiphyseal plate transfers have been developed, creation of a functional composite tissue graft with an intrinsic vascular network will prove much more difficult. Such a graft will require the precise association of multiple cell types in three-dimensions, and seems prohibitively complex now. Future solutions may incorporate combination technologies to create autologous tissue grafts \textit{in vitro}. Superstructure engineering may permit the development of numerous closely-associated polymers with varied structural arrangements\textsuperscript{235}. Incorporation of specific growth factors or inhibitors\textsuperscript{83}, coupled with selective cell seeding, may facilitate the spatial arrangement of multiple cell types. Stimulation of angiogenesis or other cellular functions at specific locations could potentially be achieved using polymer microsphere drug delivery to release cytokines or growth factors. Refinement of these technologies may permit successful generation of grafts with complex organizations of multiple cell types\textsuperscript{114}.

Until tissue-engineered grafts become available, allograft vascularized epiphyseal plate transplants from cadaveric donors remains the most promising avenue. Additional research is necessary, however, prior to clinical application in humans. With further confluence of knowledge ranging from materials science, cell and molecular biology, and experimental surgery, the prospects for successful vascularized epiphyseal plate transplantation will come ever closer to reality.
5 APPENDICES
APPENDIX A: Perfusion of vascularized proximal tibia epiphyseal plate grafts – $^{57}$Co radioactive microspheres

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>BLOOD FLOW PER 100G (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
</tr>
<tr>
<td>Unmanipulated</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
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<td>Mean</td>
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<tr>
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<tr>
<td>Island graft</td>
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<tr>
<td></td>
<td>2</td>
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<td></td>
<td>3</td>
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<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>SD</td>
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<tr>
<td>Free graft</td>
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<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>SD</td>
</tr>
</tbody>
</table>

**LEGEND:**
- **Unmanipulated** - proximal tibia epiphyseal plate without surgical manipulation.
- **Island graft** - proximal tibia epiphyseal plate graft isolated on popliteal vascular pedicle.
- **Free graft** - proximal tibia epiphyseal plate graft 48 hours after free transplantation to recipient rabbit, with microvascular anastomoses.
APPENDIX B: Immunohistochemical staining technique for BrdU in paraffin-embedded, decalcified tissue

Reagents

- Toluene
- Ethanol (100%, 95%, and 70% concentrations)
- Hydrogen peroxide 3%
- Pepsin (0.4% working solution, pH 2.0)
- PBS (pH 7.6)
- AEC (0.0125% w/v in 0.2M acetate buffer, pH 5.2)
- Crystal/Mount (dilute 1:1 with 50% ethanol)
- Mayer’s hematoxylin
- 2 N HCl

Antisera

1. Mouse anti-BrdU antibody (Caltag Lab, clone IU4) diluted 1/500 in antibody diluting buffer (Dimension Laboratories)

2. Biotinylated goat anti-mouse IgG (Zymed Lab Inc.) diluted 1/100 in antibody diluting buffer

3. Peroxidase-conjugated Streptavidin (Ultrastreptavidin kit, Signet Lab Inc.)

Method

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hydration and peroxidase blocking:</td>
<td>Time</td>
</tr>
<tr>
<td>a) De-paraffinize in toluene</td>
<td>10 minutes</td>
</tr>
<tr>
<td>b) Hydrate in serial ethanols (100%, 95%, and 70% concentrations)</td>
<td>10 minutes</td>
</tr>
<tr>
<td>c) Block in 3% hydrogen peroxide</td>
<td>10 minutes</td>
</tr>
<tr>
<td>d) Rinse in DH2O x 2</td>
<td></td>
</tr>
<tr>
<td>e) Rinse in PBS x 2</td>
<td></td>
</tr>
<tr>
<td>2. Pre-warm pepsin to 42°C in a waterbath</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Digest slides in pepsin</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Rinse slides well in DH2O</td>
<td></td>
</tr>
<tr>
<td>3. Soak slides in 2N HCl at room temperature</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Rinse well in DH2O followed by PBS</td>
<td></td>
</tr>
<tr>
<td>4. Place slides in moist chamber at room temperature</td>
<td>16 hours</td>
</tr>
<tr>
<td>Incubate with primary antibody</td>
<td></td>
</tr>
<tr>
<td>Rinse with PBS x 2</td>
<td></td>
</tr>
</tbody>
</table>
5. Place slides in moist chamber at room temperature
   Incubate with linking antibody (biotinylated goat anti-mouse IgG)
   Rinse with PBS x 2
   30 minutes

6. Place slides in moist chamber at room temperature
   Incubate with streptavidin enzyme complex
   Rinse with PBS x 2
   30 minutes

7. Add AEC substrate
   Rinse in DH2O x 2
   20 minutes

8. Counterstain with Mayer’s hematoxylin
   Rinse well with tap water
   30 seconds

9. Add 2-4 drops Crystal/Mount to damp slides and distribute evenly
    Set slides in flat metal trays and incubate at 60°C until hardened
APPENDIX C: Randomization protocol and Mycophenolate mofetil

Mycophenolate mofetil – a new immunosuppressant

Due to the known species-specific toxicity of Cyclosporine in rabbits\(^ {107}\), the immunosuppressant originally intended for use in this study was one known as Mycophenolate mofetil. This new immunosuppressant has shown promising results for both immunosuppressive efficacy and reduction in systemic side effects\(^ {195, 196}\). This drug prevents DNA synthesis via potent inhibition of inosine monophosphate dehydrogenase (IMPDH),\(^ {72}\) the rate-limiting enzyme in the \textit{de novo} pathway of purine biosynthesis. Unlike most other cell types, lymphocytes have limited ability to use the salvage pathway for synthesis of purines and therefore appear particularly susceptible to inhibition via the \textit{de novo} pathway\(^ {9}\). Mycophenolate mofetil is currently investigational and being used in clinical trials in humans receiving organ transplants.

The effects of mycophenolate mofetil are specific to B and T lymphocytes. Studies in animals and in humans have shown that mycophenolate mofetil has potent immunosuppressive properties which exceed those of cyclosporine but with reduced toxicity\(^ {143, 171, 196}\). Given that the duration of immunosuppression in this study was relatively long compared to previous studies in rabbits, an immunosuppressant lacking the species-specific toxicity of Cyclosporine A was desirable in order to maximize the chances of animal survival.

Although studies specific to rabbits have been few, the findings have been similar to those in other animal models\(^ {239}\). Mycophenolate mofetil specifically lacks the nephrotoxicity and hepatotoxicity of cyclosporine\(^ {19}\) and does not lead to the same chronic wasting syndrome in rabbits (Yatscoff, personal communication, 1996). Single-dose pharmacokinetics have been studied in rabbits, showing that mycophenolate mofetil is rapidly metabolized and excreted in the urine\(^ {238}\). The side effects of this drug are mainly gastrointestinal, including GI upset and diarrhea in some animals and humans, but has not been documented in rabbits.

Break in randomization protocol

Mycophenolate mofetil was used as the primary immunosuppressant at the beginning of this project. The principal experiment began with randomization of animals to each of the four main experimental groups described previously (TABLE 3; pg. 60), with the exception that mycophenolate mofetil was used in the immunosuppressed groups. Several months later, as the histological results became available, it was clear that the animals receiving mycophenolate mofetil were inadequately immunosuppressed. Due to time constraints, the remainder of the experiment was conducted using Cyclosporine A as the immunosuppressant, an agent with known efficacy in rabbits and previous success at our institution. Results from animals who received mycophenolate mofetil were not included in this study. The required quota of non-immunosuppressed animals had been largely completed by this time. Thus, animals were randomly allocated only to the two remaining deficient groups – specifically those requiring immunosuppression (Cyclosporine A) either with or without bone contact.
## APPENDIX D: Morphometric and histologic data for Preliminary Study B

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>MACROSCOPIC AND MORPHOMETRIC DATA</th>
<th>REJECTION INDEX</th>
<th>BrdU INDEX</th>
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</thead>
<tbody>
<tr>
<td>Rabbit</td>
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<td>Total Score</td>
<td>Average Score</td>
</tr>
<tr>
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<td>5.00</td>
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<td>graft Positive 7.45 11.14 3.69 70.00</td>
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<td>tibia Positive 35.00 43.27 8.27 0.00</td>
<td>19.00</td>
<td>4.75</td>
</tr>
<tr>
<td>7188</td>
<td>graft Positive 8.13 12.50 4.37 50.00</td>
<td>18.00</td>
<td>4.50</td>
</tr>
</tbody>
</table>

| Grafts - Mean | 7.82 | 13.07 | 5.25 | 56.00 | 12.40 | 3.10 | 14.71% |
| Grafts - SD   | 0.31 | 1.54  | 1.53 | 13.42 | 7.70  | 4.50 | 2.71%  |

| Tibiae - Mean | 34.06 | 41.24 | 7.17 | 0.00  | 19.20 | 4.80 | 13.19% |
| Tibiae - SD   | 2.81  | 1.77  | 1.82 | 0.00  | 0.84  | 0.21 | 2.80%  |

| Rabbit   | Pedicle Original Final Growth Plate | | |
| Number   | Patency size (mm) size (mm) (mm) (%) | | |
| 7136     | tibia Negative 32.35 39.64 7.29 0.00 | | |
| 7136     | graft Negative 8.12 8.27 0.15 0.00 | | |
| 7147     | tibia Negative 29.34 37.11 7.77 0.00 | | |
| 7147     | graft Negative 7.75 7.69 -0.06 0.00 | | |
| 7148     | tibia Negative 27.43 35.44 8.01 0.00 | | |
| 7148     | graft Negative 7.96 8.12 0.16 0.00 | | |
| 7149     | tibia Negative 27.72 34.97 7.25 0.00 | | |
| 7149     | graft Negative 8.04 8.11 0.07 0.00 | | |
| 7186     | tibia Negative 40.14 46.78 6.64 0.00 | | |
| 7186     | graft Negative 8.22 8.18 -0.04 0.00 | | |

| Grafts - Mean | 8.02 | 8.07 | 0.06 | 0.00 | 6.20 | 1.55 | 0.00% |
| Grafts - SD   | 0.18 | 0.22 | 0.10 | 0.00 | 1.30 | 0.33 | 0.00% |

| Tibiae - Mean | 31.40 | 38.79 | 7.39 | 0.00 | 19.20 | 4.80 | 16.30% |
| Tibiae - SD   | 5.26  | 4.83  | 0.53 | 0.00 | 0.84  | 0.21 | 1.64%  |

### LEGEND:
- **Tibia** – refers to the unmanipulated, control proximal tibia of each recipient animal
- **Original size** – refers to the size of grafts and tibiae at the time of transplantation
- **Final size** – refers to the size of grafts and tibiae at the time of sacrifice
- **Growth** – the difference between final size and original size
APPENDIX E: Morphometric and histologic data for principal experimental study

TABLE 10: Part 1 - Short-term immunosuppression with bone contact

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>MACROSCOPIC AND MORPHOMETRIC DATA</th>
<th>REJECTION INDEX</th>
<th>BrdU INDEX</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>Final Size (mm)</td>
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**LEGEND:**

Tibia – refers to the unmanipulated, control proximal tibia of each recipient animal
Original size – refers to the size of grafts and tibias at the time of transplantation
Final size – refers to the size of grafts and tibias at the time of sacrifice
Growth – the difference between final size and original size
TABLE 11: Part 2 - Short-term immunosuppression without bone contact

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>MACROSCOPIC AND MORPHOMETRIC DATA</th>
<th>REJECTION INDEX</th>
<th>BrdU INDEX</th>
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<tbody>
<tr>
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<td>Original size (mm)</td>
<td>Final Size (mm)</td>
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<td>36.24</td>
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</table>

LEGEND:

Tibia – refers to the unmanipulated, control proximal tibia of each recipient animal
Original size – refers to the size of grafts and tibiae at the time of transplantation
Final size - refers to the size of grafts and tibiae at the time of sacrifice
Growth – the difference between final size and original size
APPENDIX F: Morphometric and histologic data for continuously immunosuppressed specimens (10 weeks) both with and without bone contact

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>MACROSCOPIC AND MORPHOMETRIC DATA</th>
<th>REJECTION INDEX</th>
<th>BrdU INDEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Number</td>
<td>Pedicle Patency</td>
<td>Original size (mm)</td>
<td>Final size (mm)</td>
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<td>17.17</td>
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<tr>
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<td>14.62</td>
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<th>SPECIMEN</th>
<th>MACROSCOPIC AND MORPHOMETRIC DATA</th>
<th>REJECTION INDEX</th>
<th>BrdU INDEX</th>
</tr>
</thead>
<tbody>
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<td>17.28</td>
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<td>Original size – refers to the size of grafts and tibiae at the time of transplantation</td>
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<td>Final size - refers to the size of grafts and tibiae at the time of sacrifice</td>
</tr>
<tr>
<td>Growth – the difference between final size and original size</td>
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6 REFERENCES


47. Canafax D, Torres A, Fryd D. The effects of delayed function on recipients of cadaver renal allografts: a study of 158 patients randomized to cyclosporine or ALG-azathioprine. Transplantation 1986;41:177.


202. Starr D. Congenital absence of the radius. JBJS (B) 1945;27B:572.


