

Composite tissue allotransplantation
Functional, immunological and ethical aspects

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Composite tissue
allotransplantation
Functional, immunological and ethical
aspects

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Functionele, immunologische
en ethische aspecten
(met een samenvatting in het Nederlands)

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Chapters 1, 2, 7, 14 and 15 will be defended by M. Vossen and chapters 5, 6, 9, 10, 11 and 12 by P.C.R. Brouha. The remaining chapters will be defended by both authors.



Introduction

To graft wings of an eagle to the body of a lion, with the face and mind of the human being, is not the start of chimerism. This is the basic philosophy of plastic surgery – creativity

(N. Ben-Hur and J.M. Converse. The impact of plastic surgery on transplantation. From skin graft to microsurgery. Transplant Proc. 1980;12(4): 616-20)

INTRODUCTION

Patients with large tissue defects resulting from trauma, extirpation of tumors, major burns or congenital birth defects number in the millions costing the health-care system tens of millions of dollars each year in the United States alone. A survey conducted by Langer and Vacanti in 1993 estimated organ and tissue deficiencies in the United States¹. This survey revealed that more than 7 million people need tissue (skin, nerves, bone, cartilage, tendon or ligaments) for some type of reconstruction each year in the US alone^{2,3}. This figure is more than double the number of solid organs (heart, liver, pancreas, and kidney) needed.

The vast majority of patients requiring these tissues are currently treated by reconstructive procedures that utilize autologous tissues and/or prosthetic materials. However, the best possible outcomes are achieved when these defects are repaired using native tissue i.e. the same tissue lost to the trauma or disease. This is possible in cases of amputation due to trauma where the original tissue/body part is recovered from the scene of the accident and is reattached shortly after the injury. In these cases one can expect good recovery of function and aesthetic appearance following reconstruction if; 1) the amputated tissue is not too destroyed from the accident, 2) the time elapsed between amputation and reattachment is short and 3) the amputated tissue is cooled during the time it is ischemic (between amputation and reattachment). Unfortunately, in the majority of cases the original tissues are not available to be used for reconstructing these defects. This is because more often than not the above three criteria are not met or the original body part is destroyed (invaded by cancer, crushed by trauma beyond use or severely burned) or did not exist in the first place (congenital birth defects). In the absence of the native body part/tissue (the majority of cases) surgeons must reconstruct these defects using autologous tissues and/or prosthetic materials. These reconstructive procedures consist of transferring one or combinations of several tissues from another part of the patients' own body to repair these defects. In the event the patients' own tissues do not suffice to reconstruct a given defect a variety of different prosthetic material constructs are also available for this purpose. Though these procedures have advanced a great deal over the years they are still plagued by many drawbacks and their functional and aesthetic outcomes still do not come close to those achieved by procedures that use the native body part/tissue for reconstruction.

Limitations of currently used reconstructive procedures include; poor functional and aesthetic outcomes; multiple (often 10 to 15) procedures to revise the original surgery; prolonged rehabilitation resulting in patients not

returning to work or normal life and becoming dependent on family members and the health care system for care; high costs of multiple surgeries/hospitalizations; donor site morbidity resulting from use of autologous tissues and post operative complications associated with implanted prosthetic materials "foreign body" (infection, altered healing, rejection, etc.).

One potential solution to this great need for native tissue is composite tissue allotransplantation (CTA). As solid organ transplantation revolutionized the treatment of terminal organ failure, CTA could fulfil the existing great need for native tissues to reconstruct large tissue defects. Although in a few isolated clinical cases tissues/structures (nerves, bone, joint, muscle, larynx, entire hands, and partial face) have been transplanted from donors⁴, CTA has not yet gained widespread clinical use. This can be attributed to one main reason; the risks posed by the immunosuppressive drugs required to prevent rejection are considered by many to be too high a price to pay for the benefits a patient would receive from one of these non-life-threatening reconstructive procedures using a CTA.

A safe alternative to presently used (in solid organ transplantation) non-specific "drug based" immunosuppression could provide a solution to this risk vs. benefit argument. Generating donor specific tolerance through mixed allogenic chimerism (MAC) has potential for being a safe alternative to immunosuppressive drugs⁵⁻⁷. To date donor specific tolerance for solid organ or bone marrow transplantation has been achieved through MAC in small and large animal models. However, as with drug based immunosuppression MAC also carries with it associated risks i.e. the risk of developing graft versus host disease (GVHD) associated with the bone marrow engraftment necessary to generate MAC⁸⁻¹¹.

Even though much progress has been made using autologous tissues and prosthetic materials, still the best functional and aesthetic outcomes are achieved when tissue(s) whose native form and function are most similar to the missing tissue(s) are used. Both functionally and aesthetically the patient could return to work and a normal life in a very short time. The ability to use CTA in complex reconstructions eliminates most of the above listed limitations (poor functional and aesthetic outcomes, multiple revision surgeries, prolonged rehabilitation, high costs, donor site morbidity and foreign body associated complications) and in doing so revolutionizes the field of reconstructive surgery.

COMPOSITE TISSUE ALLOTRANSPLANTATION

The concept of transplanting composite tissue in the form of a limb is not new. In a painting from the 15th century the Saints Cosmas and Damian are shown replacing a Caucasian's amputated leg with one taken from a recently deceased Moor^{12,13}. In more recent literature, reconstructive surgeons have reported the development of new instrumentation, techniques and an improved knowledge of tissue pathophysiology that today allows them to successfully reattach amputated fingers, hands, feet and even entire limbs¹⁴. Using these same techniques they routinely transfer tissues from one part of the body to another to reconstruct any part of the human anatomy. In addition to using the patients' own tissues to reconstruct large tissue defects, reconstructive surgeons have recently performed allotransplantations using tissues from cadavers or brain dead, heart beating donors. Prior to clinical implementation, these CTA procedures have been extensively investigated in various animal models, such as a rat models^{15,16} and a pre-clinical porcine model¹⁷. Hovius et al. investigated the feasibility of partial hand allotransplantations in rhesus monkeys¹⁸. More recently animal models for facial transplantation have been developed in rats¹⁹⁻²¹ and canines²².

To date a total of 62 clinical composite allotransplantations have been reported in literature (Table 1)⁴.

Table 1. Clinically reported CTA procedures in chronological order.

CTA transplantations	Number of transplants
Hand	25
Trachea	3
Tendon	2
Knee and femoral diaphysis	8
Muscle	1
Larynx	1
Nerve	7
Abdomen	9
Tongue	1
Scalp	2
Face	3
Total	62

The first human hand transplantation was performed in 1963 by a team of surgeons in Ecuador, however the immunosuppressive regimen at that time (azathioprine and hydrocortisone) was inadequate and the hand rejected within three weeks, followed by amputation^{23,24}. Finally, in 1998 a team of surgeons in Lyon, France²⁵ and early 1999 a surgical team in Louisville, Kentucky, USA²⁶, performed hand transplantation in two patients. In 1979, Rose and colleagues reported a transplantation of a trachea using a two stage procedure²⁷. Thereafter, two more reports of trachea transplantation have been published^{28,29}. In the early 1990s Guimbertau and colleagues reported the first allotransplantation of two vascularized digital flexor tendon apparatus³⁰. Soon thereafter Hoffman and colleagues performed between 1994 and 2000 three vascularized femoral diaphysis and five whole knee joint allotransplantations in patients that sustained bone defects resulting from tumor resection and trauma³¹. In 1998, a vascularized latissimus dorsi muscle allotransplant was used to cover a large tissue defect in the scalp region in a kidney transplant patient³². A single clinical case of a larynx allotransplant to restore voice function was reported by Strome³³. Furthermore, vascularized nerve allografts have been successfully transplanted in the clinical setting to restore denervation in the extremities³⁴. Abdominal wall allotransplantation has been performed after visceral transplantation for coverage in nine patients. The allografts included one or both rectus abdominus muscles, fascia, subcutaneous tissue and skin^{35,36}. Furthermore, in 2003 Birchall successfully performed a tongue transplantation in a patient who had tongue cancer³⁷.

Up to date two scalp transplantations have been reported^{38,39} and the most recent revolution in CTA is the first successful partial face transplantation including nose and lips performed in Amiens, France in November 2005 (see figure 1, upper panel)^{40,41}. In addition, two more partial face transplantations appeared in the press. In 2006, a bear attack victim in China received a partial face allograft that included cheeks, lips, nose and chin from a brain-dead donor⁴². In 2007, Lantieri and colleagues in Paris, France performed the third partial face transplantation, which included nose, mouth and chin, in a man that suffered from von Recklinghausen disease⁴³ (personal communication, see figure 1, lower panel).

The outcomes of the above transplant procedures have been generally successful using conventional immunosuppressive drug regimens to prevent rejection.



Figure 1. Partial face transplantation.

Upper panel. The first successful partial face transplantation including nose and lips performed in Amiens, France in November 2005. *Left.* Preoperative view. *Right.* One-year postoperative view. Photographs courtesy of B. Lengelé, MD, PhD⁴¹.

Lower panel. The third partial face transplantation including nose, mouth and chin, in a man that suffered from von Recklinghausen disease. *Left.* Preoperative view. *Right.* Postoperative view. Photographs courtesy of L. Lantieri, MD, PhD.

METHODS OF IMMUNOMODULATION RELEVANT TO THIS THESIS

Immunosuppressive drugs have revolutionized clinical transplantation⁴⁴, to the point where today solid organ transplants are considered to be “standard of care” for patients suffering with end organ failure. In spite of this success, even the most modern immunosuppressive drugs used today bring with them significant risks, including an increased incidence of neoplasm, opportunistic infections, and end organ toxicity, such as drug induced bone loss⁴⁵⁻⁵¹. The mechanism by which these immunosuppressive drugs cause the above risks is closely linked to the nonspecific mechanism by which they prevent allograft rejection^{49,52}.

Corticosteroids have remained the basic drug used in all whole organ transplantations since the early 1950's. Prednisone, the prototypic agent used, is analogous to the major endogenous corticosteroid, cortisol (hydrocortisone). However, it is four times more potent in efficacy than cortisol. The actions of corticosteroids are mediated by subcellular hormone receptors that form steroid receptor complexes. These complexes bind to DNA and affect expression of specific genes that drive proteinsynthesis and cellular processes. Corticosteroids were initially used to reverse acute rejection of the transplant. Currently, it is common practice to use much-reduced doses of corticosteroids in combination with other drugs for maintenance therapy or short courses of high doses for treatment of acute rejection. Corticosteroids form a powerful toxic influence on bone. This has been shown even in previously healthy individuals in whom the adverse effects of glucocorticoids are considerable.

Cyclosporine A (CsA) is the prototype agent in the class of calcineurin inhibitors. This specific group of immunosuppressants continues to be the cornerstone of successful long-term immunosuppressive regimens. They exert their effects through regulation of cytokine production. The introduction of CsA was one of the first major advancements in transplantation since the release of corticosteroids during the early 1950's.

Tacrolimus (FK506) is also a calcineurin inhibitor. In its actions on the immune system tacrolimus resembles CsA, but it is structurally distinct from CsA and has been shown in vitro to be 100 times more potent. The introduction of FK506 during the 1980's improved outcomes in organ transplantation. FK506 favourably affects nerve regeneration. In numerous vitro and in vivo studies FK506 was found to reduce the time to neurological re-

covery following a nerve lesion due to enhanced rates of axon regeneration. Furthermore, FK506 doubles the number of axons that regenerate following a nerve injury and significantly increases myelin thickness⁵³. This effect will benefit functional outcome of a CTA.

The calcineurin inhibitors have complex and incompletely understood actions on bone. Experimentally, in rat studies, CsA accelerates bone resorption and leads to a high turnover osteopenia. FK506 shows effects on bone in rat models that are equivalent to those of CsA, however clinically, these effects have been uncertain. Furthermore it is not always possible to distinguish between the effects of the calcineurin inhibitor and those of corticosteroids.

Combination therapy: Regardless of how drugs affect immune function, no one drug possesses a level of efficacy and a margin of safety that it can be used alone as monotherapy. Many immunosuppressive drugs have different mechanisms of action and non-overlapping toxicities. Therefore they can be administered together in a variety of different combinations to achieve powerful immunosuppressive effects and at the same time minimal toxicity. This form of therapy is known as combination therapy^{44,46}. This merger of high effectiveness and low toxicity provided by combination therapy was applied successfully in solid organ transplantation. Based on this experience the first clinical cases of CTA were performed using a FK506 based combination therapy. In spite of encouraging clinical results achieved with combination therapy, there continues to be considerable debate as to whether the risks associated with the use of these chronic non-specific methods of immunosuppression justify the benefits of using composite tissue allotransplantation to treat non-life-threatening reconstructions. A possible alternative to using chronic non-specific immunosuppression is to create a stable state of tolerance to the transplanted tissue i.e. chimerism.

Chimerism refers to the coexistence of cells from two genetically distinct organisms in one individual^{54,55}. In hematopoietic stem cell chimerism, the cells that coexist are derived from the immune systems of the host and/or donor through a bone marrow transplant, thus producing tolerance to donor tissue in the host. Having achieved this tolerance, conventional immunosuppressive drugs are not necessary to prevent rejection of donor tissue^{6,56}. There are different types of chimerism as it relates to bone marrow transplantation: *syngeneic chimerism* refers to when a host receives genetically identical donor bone marrow cells. *Allogeneic chimerism* refers to

when a host receives genetically disparate donor bone marrow cells. Allogeneic chimerism can be subdivided into *fully allogeneic chimerism* and *mixed allogeneic chimerism (MAC)*. In the former the host receives 100 % genetically disparate donor bone marrow cells while in the latter, the host receives only part genetically disparate donor bone marrow cells. In MAC these transplanted donor bone marrow cells co-exist with the host's own bone marrow cells, whereas in fully allogeneic chimerism the donor system fully replaces the recipient hematopoietic system^{57,58}.

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Aims and Outline

The first clinical composite tissue allotransplantations (CTAs) in the form of hand, larynx, and face transplantations have recently been performed. However, for this new reconstructive method to gain widespread acceptance and to become standard care important aspects need to be examined.

Rejection of the current CTAs is prevented by toxic immunosuppressive drugs. As a result ethical dilemmas related to risk versus benefit in these new and experimental procedures need to be objectively investigated for the various transplant procedures. Furthermore the long-term function of a CTA as related to bone integrity is unclear and needs more research. In the future we will almost certainly move away from toxic immunosuppressive drugs and prevent rejection by induction of immunological tolerance. Therefore we need to address the immunological challenges involved and to minimize its possible side effects.

The studies described in this thesis were designed to address these important aspects and to aid introduction of CTA into common medical practice, ultimately creating a new and exciting era in plastic and reconstructive surgery. Therefore this thesis is divided into three parts.

In **PART I** different combinations of immunosuppressive regimens are studied in both a porcine and rat model. In particular the effect of the immunosuppressive regimens on bone quality was investigated.

In **chapter 1** a cyclosporine A (CsA) based combination immunosuppressive regimen was studied in a porcine composite tissue allotransplantation model. This regimen of CsA, Mycophenolate Mofetil (MMF) and prednisone was at the time commonly used in vascularised bone and joint allotransplantation. Bone quality was studied pre- and post-transplant by measuring acoustic velocity and density. Furthermore bone healing was assessed.

Since the patients that received human hand transplants all used Tacrolimus (FK506), MMF and prednisone immunosuppressive therapy, this regimen was investigated in **chapter 2**. Bone quality and healing were studied pre- and post-transplant in a porcine CTA model. In addition, bone quality analyses were performed in bones from non-operated limbs and in bones from autograft procedures to look at the effect on bone quality of the transplant procedure itself.

Corticosteroids are known to have a detrimental influence on bone. However, in the clinical situation of allotransplant patients using combination immunosuppressive regimens, it is difficult to distinguish between the effects of the calcineurin inhibitors, such as FK506, and those of corticosteroids. Therefore, at first in **chapter 3** we describe a study to determine

whether a low dose, corticosteroid-free combination regimen of FK506 and MMF would prevent rejection in a rat composite tissue allotransplant model. Secondly, in **chapter 4**, bone quality and healing is studied in this same animal model in which hind limb CTA was performed. Bone quality was studied pre- and post-transplant by measuring acoustic velocity and density.

In **PART II** chimerism is studied as a way to induce tolerance in composite tissue allotransplantation recipients. Especially the role of graft-versus-host disease (GVHD) is studied in chimeric recipients of CTA. **Chapter 5** reviews the use of bone marrow transplantation for induction of chimerism and donor-specific tolerance with special emphasis on approaches to overcome the current limitations. When transplanting rat hind limbs to tolerant chimeric hosts, the mature T-cell content of these limbs induced lethal GVHD in 100% of animals. In **chapter 6** we demonstrated that inactivating these cells with irradiation prevents GVHD, destabilization of chimerism, and permits rejection free graft acceptance. In **chapter 7** a clinically feasible protocol is introduced for allotransplantation. We eliminated the delay period in a rat hind limb allotransplantation model by performing mixed allogeneic chimerism induction and transplantation simultaneously. Ever since we saw GVHD in chimeric hosts after hind limb transplantation, we were interested which lymphocytes within the hind limb caused lethal GVHD. Therefore, we designed a study (**chapter 8**) to determine in chimeric hosts whether the lymphocytes within the bone marrow and/or lymph nodes (LNs) transplanted with the limb cause GVHD. From this study we concluded that mixed chimeras are susceptible to GVHD when receiving LN bearing grafts. Following these results, in **chapter 10** we tried to establish the role of the cellular fraction versus the microenvironment of LNs in the development of GVHD in our chimeric model. However to succeed in this aim, we first needed to establish a simple and applicable model for LN transplantation, which is described in **chapter 9**.

In **PART III** we investigate risk acceptance and focus on ethics in composite tissue allotransplantation. The introduction of facial transplantation in the clinical arena in November 2005 made the ethical debate on risk versus benefit in composite tissue allotransplantation procedures, especially facial transplantation, even more meaningful.

In **chapter 11** a questionnaire-based instrument, aimed to objectively assess the relative risk that individuals are willing to accept in order to receive the benefits of various composite tissue allotransplantation procedures, is described and validated.

In **chapter 12** this questionnaire-based instrument was utilized to quantitatively assess the amount of risks that individuals are willing to take to receive the benefits of composite tissue allotransplantation procedures. Two populations of individuals were studied: 1) those who live with the risks of immunosuppression and 2) healthy individuals. In addition, a comparison between the two groups was made.

In **chapter 13** the investigation of risk acceptance in composite tissue allotransplantation procedures is further specified to facial transplantation. Three study populations (healthy individuals, organ transplant recipients, and individuals with facial disfigurement) were questioned to evaluate the degree of risk that individuals are willing to accept for, among others, a facial transplant, especially those who could directly benefit from facial transplantation.

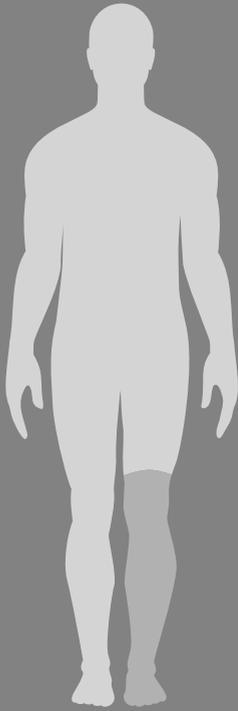
The purpose of **chapter 14** is to provide information on the major technical, immunological and ethical issues surrounding facial transplantation.

The field of transplantation surgery has always pushed the boundaries of medicine forward and in doing so it repeatedly raised unprecedented ethical questions. In **chapter 15** the many complex and extra-ordinary ethical issues that arise with the introduction of human facial transplantation are discussed. Furthermore in this chapter, criteria are developed, that we maintain, must be satisfied in order to ethically undertake this innovative transplant procedure.



Part





Chapter 1

Bone quality and healing in a swine vascularized bone allotransplantation model using cyclosporine-based immunosuppression therapy

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INTRODUCTION

In spite of the many advances that orthopedic and trauma surgery have experienced in recent years, reconstruction of large bony defects, especially of long bones and joints continue to pose a major problem. The most advanced methods used for treating these large bony defects consist of bone replacement with vascularized autologous bone transfers¹, non-vascularized allogeneic bone transfers² and/or bone replacement using prosthetic materials. While these methods of replacing bony tissue have greatly improved clinical outcomes in these procedures there remains much room for improvement. Some of the more important drawbacks of current methods are the scarce supply of donor tissue, donor site morbidity, inability to include articular cartilage and supporting ligaments into the graft and the fact that the current methods are often accompanied by complications such as infection or non-union³⁻⁵.

A treatment approach that could solve many of these problems is replacing these large bony defects with like tissue from human donors as is done in solid organ transplantation. The concept of transplanting vascularized bone grafts from allogeneic donors is not new and has been considered and investigated for many years in animal models⁶⁻¹³. More recently, in a few select clinical cases, Hofmann et al. used the concept of transplanting vascularized bone from brain dead, heart-beating donors to reconstruct large bone and joint defects¹⁴⁻¹⁷. The obvious downside of using vascularized bone allografts is the necessity to use immunosuppressive drugs to prevent rejection. Many recent studies focusing on the use of vascularized bone allografts have employed cyclosporin A (CsA) alone or in combination with other drugs as the immunosuppressant therapy. The results of these studies have been promising, reporting high levels of effectiveness in preventing allograft rejection^{8,18}. While CsA has been effective in preventing bone allograft rejection little is known about its effects on the structural and material properties of bone and bone healing. Since bone quality and healing are crucial to the long-term functional success of vascularized bone and/or joint allografts, we designed the present study to assess the effects a commonly used CsA based immunosuppressant regimen has on bone quality and healing in a pre-clinical swine osteomyocutaneous allotransplantation model.

MATERIALS AND METHODS

Radial forelimb free flaps containing bone, muscle and skin (osteomyocutaneous free flaps) were transplanted between age- and size-matched, genetically mismatched pigs and the recipient animals received CsA-MMF and prednisone combination immunotherapy for 90 days. During the 90-day protocol allograft rejection was assessed by visual inspection of the flap's skin and histopathologic examination of skin biopsy specimens. Bone quality was assessed pre- and post-transplant by measuring the acoustic velocity and density of a portion of the radial bone component of the flap. Bone healing was assessed using radiographic analysis of serial radiographs.

Animal care: Twenty-two age- (8-10 weeks old) and size- (17-24 kg) matched outbred farm pigs were used in this study and cared for in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine. Donor and recipient animals were purchased from different farms to assure mismatch at the HLA alleles. Animals were housed in separate cages in light (12hr-12hr), temperature (22° C) and airflow-controlled rooms. Animals were fed standard diets and were provided with water *ad libitum*. After an initial physical examination, baseline laboratory tests were performed (complete blood cell count with differential, electrolyte levels, and liver function tests) to assess each animal's general health. Pre-transplant cross matching was performed for each donor-recipient pair to assure disparity at the HLA alleles and avoid hyperacute rejection. Animals had no prior history of allo-sensitization. At the end of the 3-month experimental protocol animals were euthanized with 6 ml of Beuthanasia® (Schering-Plough Animal Health Corp., Kenilworth, NJ).

Pig forelimb osteomyocutaneous flap allotransplant model: A detailed description of this flap model can be found elsewhere in a separate publication dedicated to this topic¹⁹. Below a brief description of the flap is provided to give the reader a general understanding. Orthotopic allotransplantation of right radial forelimb osteomyocutaneous free flaps was performed (Figure 1). The flap was designed according to the well-established concept of a radial forearm osteomyocutaneous free flap used clinically for reconstructing multi-tissue defects. Donor flaps and their respective recipient beds were prepared simultaneously by two surgical teams.

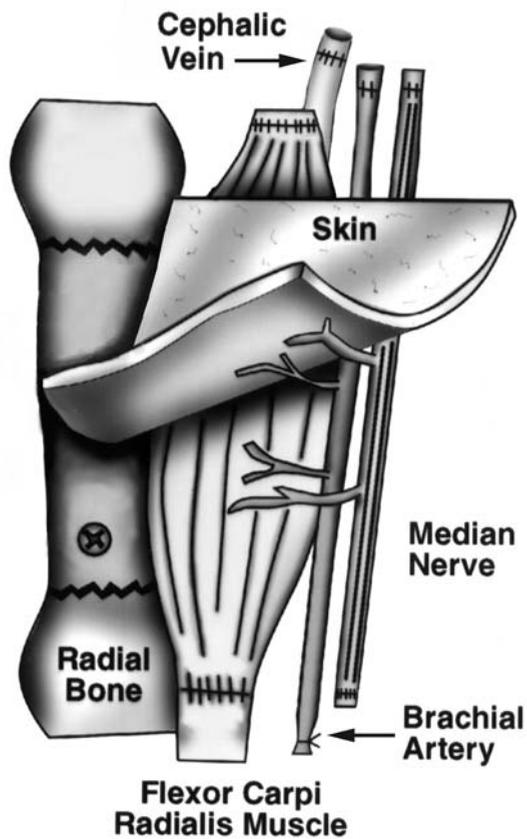


Figure 1. Schematic diagram of porcine extremity CTA model of radial forearm osteomyocutaneous flap. Skin, neurovascular, muscular, and radial bone are depicted.

Donor flap. A 6 × 6-cm area of skin over the anteromedial aspect of the pig's right forelimb was marked 3 cm below the elbow joint. After the skin incision was made, the brachial artery, cephalic vein, median and radial nerve, and attachment sites of the flexor carpi radialis were identified and dissected. Proximal and distal osteotomies were performed in the radius and a 6-cm segment of radial bone was removed from its attachment to the ulna. The principal neurovascular pedicle supplying the donor flap was left intact until the recipient bed was ready.

Recipient bed. Identical incisions and dissections were performed on the right forearm of the recipient animal as described above. Special attention was paid to hemostasis.

Transplant. Upon transferring the donor flap to the recipient bed, the donor segment of the radial bone was placed into the recipient defect and secured there with one 25- to 30-mm stainless steel screw placed into the adjacent ulna. The donor flexor carpi radialis was extended to its original length and sutured into its recipient bed. The donor vessels were anastomosed end to end to their recipient counterparts, and the flap was allowed to perfuse. The median and radial nerves were then coapted to their recipient counterparts. Finally, the wound was irrigated and examined for bleeding, and the overlying skin was closed. After skin closure, a loose sterile dressing was applied to the wound over which a fiberglass cast was placed on the limb. A window was created in the cast over the skin portion of the flap to permit daily inspection and regular biopsies of the graft until the cast was removed 3 weeks post-transplant.

Experimental groups and immunosuppression protocol: Ten osteomyocutaneous allotransplants (experimental group) and 2 sham operations (controls) were performed using 22 animals. The allotransplants were performed as described above. The 2 controls consisted of raising 2 radial forelimb osteomyocutaneous free flaps and re-implanting them back to their original location in the same animals. All allograft flap recipients received oral CsA, Mycophenolate mofetil (MMF), and prednisone combination therapy. CsA (Sandimmune® oral solution, Sandoz Pharmaceuticals, East Hanover, NJ) 40mg/kg/d was begun on the morning of surgery, with subsequent doses adjusted to maintain 24-hour whole-blood trough level between 100 and 300 ng/ml by Emit® 2000 Cyclosporine Specific Assay (Behring Diagnostics, Cupertino, CA). Oral MMF 500mg per day was begun on the morning of operation. Methylprednisolone (500mg) was administered intravenously during the procedure just before flap transfer. Animals received oral prednisone (2.0mg/kg/d) on the first postoperative day, which was then tapered by 0.5 mg/kg/d every 3 days to a maintenance dose of 0.1 mg/kg/d after 1 month. Animals in the treatment group did not have access to food during the night to increase the likelihood of their ingesting and absorption of the immunosuppressive drugs, which were mixed with small amounts of food in the morning. Finally, as per study protocol drug doses were maintained constant and were not adjusted according to clinical signs of rejection.

Postoperative care: For infection prophylaxis procaine penicillin G 30,000 units/kg, was administered intramuscularly to all recipient pigs for 10 days. Animals were also given buprenorphine (Buprenext, 0.3 mg) intramuscularly for pain management. Complete blood cell counts with differentials were determined 3 times per week for the first week, thereafter they were obtained weekly together with electrolyte levels and liver function tests. Twenty-four hour CsA trough levels were determined daily for the first 3 weeks, 3 times per week for the next 3 weeks, and then weekly thereafter. All recipients were followed up for 3 months for the occurrence of acute rejection, graft loss, or death. Rejection was assessed clinically by daily visual inspection of the flap skin by 2 examiners. Skin biopsies were performed on days 0, 2, 4, 7, 10, 14, 21, 30, 45, 60, and 90. The skin biopsy specimens were initially fixed in 10% buffered formaldehyde and then transferred to and stored in 70% ethyl alcohol. For analysis the tissue sections were stained with Hematoxylin and Eosin stains. Visual and histologic scoring systems, that were formulated in our previous work with rejected CTAs in pigs, to grade the severity of rejection were applied to the current study²⁰. Complete graft rejection, disease of the animal leading to death, or the end of the 3-month follow-up period were considered study endpoints. At the end of the experiment complete autopsies were performed on all animals.

Histopathologic assessment of bone rejection: At the time of autopsy, one cm bone specimens from mid-diaphysis of the transplanted radii were harvested for histological assessment of bone rejection. The bone specimens were fixed in 10% formaldehyde and processed to standard 4 μ m paraffin sections and stained with hematoxylin and eosin. Slides were evaluated in a blind fashion using the following criteria: mononuclear cell infiltration, fibroblastic proliferation, irregular cortical thickening, nonviable trabeculae, intratrabecular fibrosis, filigree osteoid formation and hemorrhage/ necrosis.

Assessment of bone healing: After adequate sedation, antero-posterior and lateral radiographs were taken of the transplanted radius twice during the first week, monthly thereafter and prior to sacrifice with standard 11 x 14 inch film-plates at a 40 inch film-to-tube distance at 50 kV and 4 mAs (OEC-Diasonic X-ray Imaging Systems; Salt Lake City, UT). Radiographs were graded by the modified radiologic Weiland scoring system in Table 1²¹.

Table 1. Modified Weiland scoring system for radiographs.

Grade	Proximal junction/ Distal junction	Body of the graft
0	Complete resorption/ pseudarthrosis	Complete resorption/ pseudarthrosis
1	Severe resorption	Severe resorption
2	Mild resorption	Mild resorption
3	Resembling postoperative	Resembling postoperative
4	Early union	Mild new (+2mm)
5	Solid union	Moderate new bone (+4mm)
6	Beginning remodeling	Beginning remodeling

Assessment of bone quality: Bone quality was evaluated by calculating the bone biomechanical elastic properties with data obtained from acoustic velocity and bone density measurements of the radius pre- and post-transplant²²⁻²⁶. The acoustic velocity was measured using a longitudinal ultrasound transmission technique and bone density was measured using Archimedes' principle^{27,28}. Bone specimens of approximately 1 cm were cut out of the radius bone grafts. For measurement of the acoustic velocity each specimen was placed between ultrasound transmitting and receiving transducers (Panametrics, SmH2; Waltham, MA). A square wave signal was applied to the transmitting transducer. By comparing the onset of the input and the output of this signal, the transmission time, Δt , of the ultrasound energy crossing through the specimen could be determined. The acoustic velocity (v) was then calculated as follows: $v = l/\Delta t$, where l is the length of the specimen spanning the ultrasound transducers. Results of acoustic velocity measurements were expressed in m/s.

Bone density measurements were obtained according to Archimedes' principle. The bone specimens previously used in the acoustic velocity measurements were hydrated and weighed both in and out of a water bath. Bone density measurements were calculated as follows: density (ρ) = $(A/A-B) \times P$, where A is the weight of the hydrated bone, B is the weight of the hydrated bone submerged in water, and P is the density of distilled water at a given temperature. $A-B$ is the equivalent to the volume of the bone specimen. Results of bone density measurements were expressed in kg/m^3 .

In order to further assess the potential differences in bone quality of the radius specimens pre- and post-transplant, a longitudinal elastic coefficient (E) was calculated for each bone specimen using the relationship: $E = \rho \times v^2$. Where ρ is the bone specimen density and v the measured acoustic veloci-

ty. Results of the calculated elastic coefficient were expressed in GPa. Assuming that the acoustic wave pathway in bone is homogenous, the elastic coefficient represents the intrinsic longitudinal stiffness. Although no clear quantitative relationship exists between the elastic coefficient or modulus of bone and its absolute mechanical strength^{24,28,29}, recent studies show a relatively strong correlation between its modulus and ultimate strength³⁰.

Statistical analysis: Data analysis for the biomechanical tests included single factor analysis of variance (ANOVA) followed by post-hoc paired t-test. Differences were considered significant at $p < 0.05$. Data are reported as means and standard errors of the mean.

RESULTS

Animals: All animals recovered from anesthesia without problems and were able to stand, eat and drink immediately after the recovery. None of the radial forelimb flaps failed as a result of technical problems associated with free tissue transfer and there was no morbidity attributed to the transplant procedure. Serum electrolytes, liver function tests, white blood cell and platelet counts remained within normal limits in all animals throughout the study except during times of infection. The 2 control animals recovered from surgery and the 3-month follow up period without complications. Four pigs were excluded from the bone measurements because of graft rejection or death prior to the end of the study. Of the remaining six pigs, two developed early transient grade I/II rejection lasting for almost 2 weeks. Other than this these pigs remained rejection free for the remainder of the study. Three other pigs presented with late grade I/II rejection in postoperative week 6, 8 and 13 respectively. In these three cases, the rejection persisted without progression in severity throughout the 90-day follow-up period. All animals gained bodyweight after transplantation (19.3 ± 0.7 pre-transplant vs. 28.3 ± 2.0 post-transplant).

Bone rejection: Histopathologic evaluation of the bone revealed mild rejection by mononuclear cells infiltrating the bony trabeculae and mild vasculitis without nonviable trabeculae, intratrabecular fibrosis, filigree osteoid formation, or hemorrhage/ necrosis in five samples (Figure 2). Moderate rejection as characterized by lymphocytic infiltration with areas of hemorrhage and necrosis was observed in one sample. Areas of nonvi-

able cortex were surrounded by newly formed bone. Severe and total rejection of the bone was not observed.

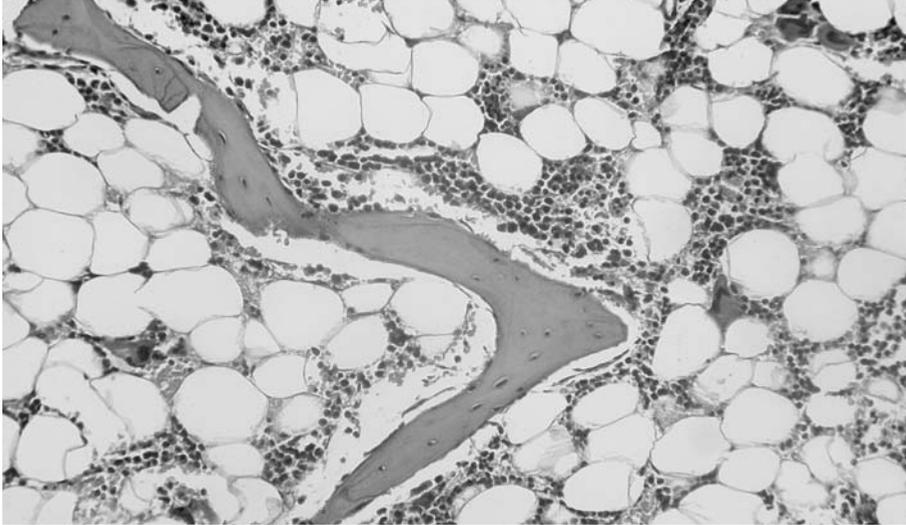


Figure 2. A: Histopathologic view of non-rejecting bone from contralateral limb showing normal bone with normal distribution of fat and hemapoetic elements. (hematoxylin and eosin; original magnification, 100X)

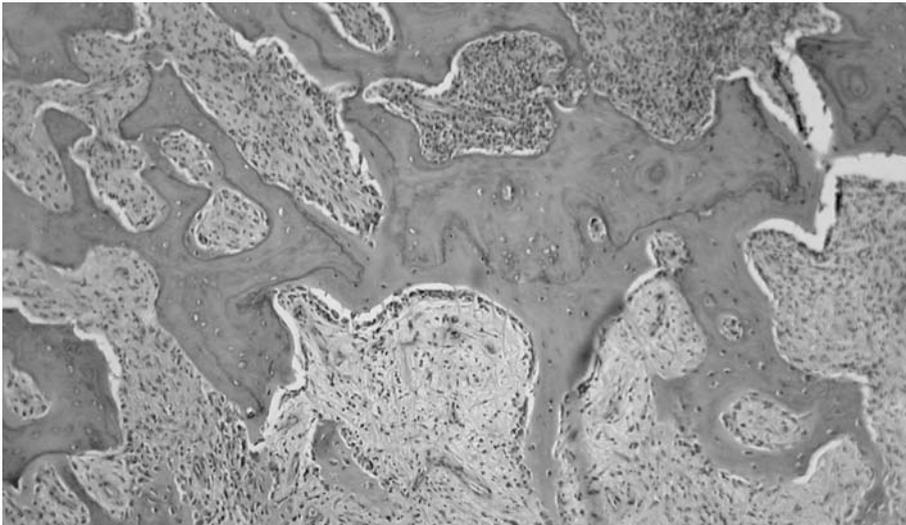


Figure 2. B: Histopathologic view of bone from osteomyocutaneous allotransplant showing mild rejection by mononuclear cells infiltrating the viable bony trabeculae. (hematoxylin and eosin; original magnification, 100X)

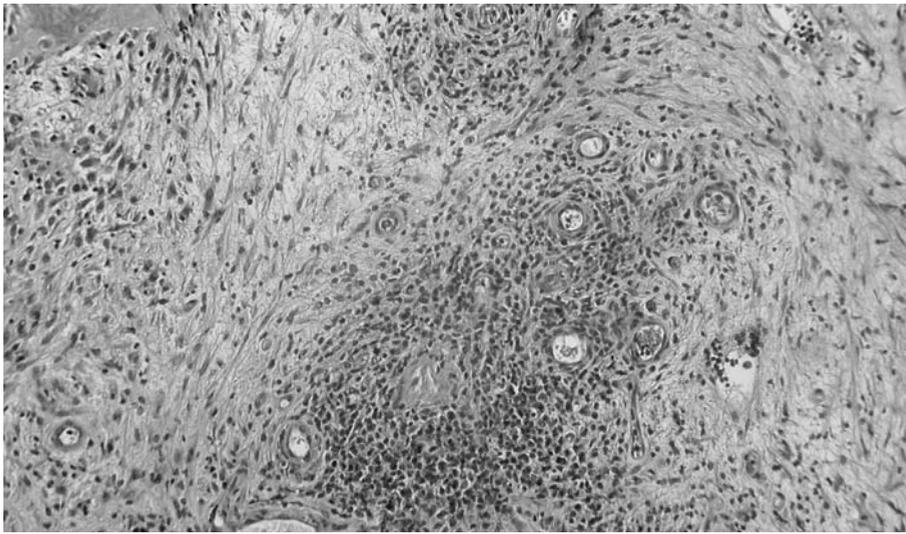


Figure 2. C. Histopathologic view of bone from osteomyocutaneous allotransplant showing vasculitis as characterized by the small artery in the center with thickening of the wall surrounded by intense mononuclear cell infiltrate. (hematoxylin and eosin; original magnification, 200X)

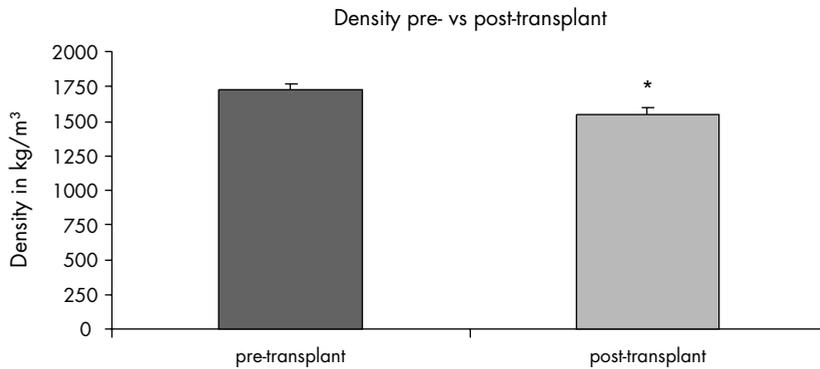


Figure 3. Graph showing the mean density of the radius before and after transplantation. Results in mean \pm SEM, $n=6$, * $p < 0,05$.

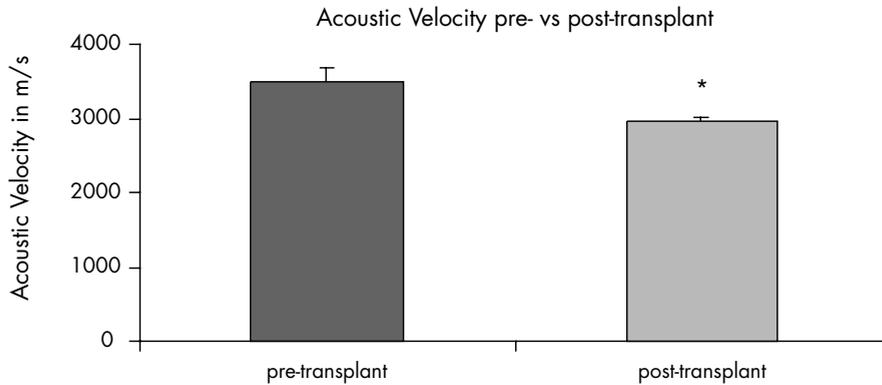


Figure 4. Graph showing the mean acoustic velocity of the radius before and after transplantation. Graph showing the mean acoustic velocity of the radius before and after transplantation. Results in mean ± SEM, n=6, * p < 0,05.

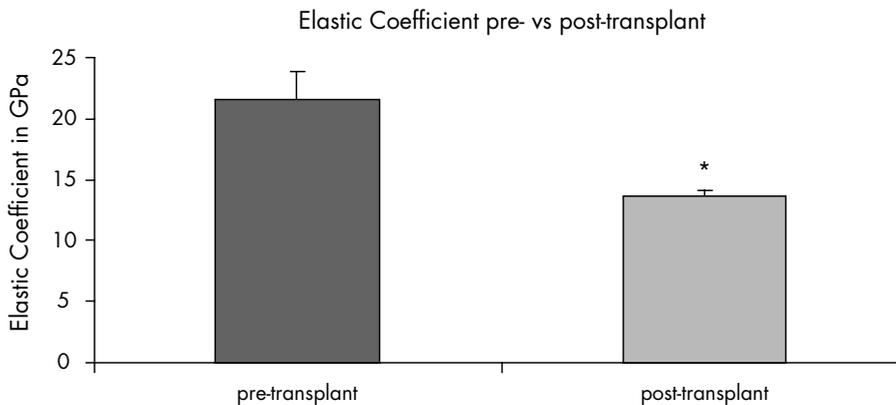


Figure 5. Graph showing the mean elastic coefficient of the radius before and after transplantation. Results in mean ± SEM, n=6, * p < 0,05.

Bone quality: The results of the bone density, velocity and the calculated elastic coefficient (bone stiffness) are summarized in Table 2 and Figures 3,4 and 5. The mean bone density pre-transplant $1722.7 \pm 44.1 \text{ kg/m}^3$ was significantly higher than the mean bone density post-transplant $1544.7 \pm 47.5 \text{ kg/m}^3$.

The difference between the mean acoustic velocity pre-transplant $3503.0 \pm 165.1 \text{ m/s}$ and post-transplant $2963.0 \pm 54.6 \text{ m/s}$ was significant. The

mean elastic coefficients pre-transplant 21.6 ± 2.2 GPa was also significantly higher than post-transplant 13.6 ± 0.5 GPa. These reductions post-transplant of density, acoustic velocity and elastic coefficient indicate that bone quality of the transplanted radii's was significantly diminished post-transplant.

Table 2. Result summary of bone density, velocity and elastic coefficients pre- and post-transplant (in mean \pm SEM, n=6 , * p < 0,05).

	Density (kg/m³)	Acoustic Velocity (m/s)	Elastic Coefficient (GPa)
Pre-transplant	1722.7 \pm 44.1	3503.0 \pm 165.1	21.6 \pm 2.2
Post-transplant	1599.4 \pm 57.7 *	2962.9 \pm 54.6 *	13.6 \pm 0.5 *

Bone healing: The radiographic results of the transplanted forelimbs show that there was bone union at the proximal and distal junctions of the transplanted radii. The progression of bone union reached grade 5-6 at the endpoint of the study. (Table 3) Furthermore, there was progressive hypertrophy in the body of the grafts reaching grade 5-6 at the endpoint of the study. There was no radiologic evidence of bone resorption or pseudoarthrosis present in any of the animals. These results were supported at autopsy when gross examination of the radius revealed the grafted bone to be hypertrophic and well incorporated with the recipient radius. The osteotomy sites could not be detected by gross visual inspection.

Table 3. Results of radiological findings of radial forelimb free flap in experimental (n=6) and control pigs (n=2). Radiographs were scored using the Modified Weiland scoring system.

Pig No.	Proximal Junction	Distal Junction	Body of the Graft
1	6	5	6
2	5	5	5
3	5	5	6
4	5	6	5
5	5	6	5
6	6	6	6
Control 1	6	6	6
Control 2	6	6	6

DISCUSSION

The concept of using vascularized bone and joint allografts to reconstruct large skeletal defects dates back in the literature to 1968³¹. However, the first experimental models of vascularized bone and joint allotransplantation were unsuccessful due to the ineffectiveness of the immunosuppressive regimens available at the time^{32,33}. In 1976, the introduction of cyclosporine A to the arsenal of immunosuppressant drugs marked a new era in transplantation³⁴. Soon, cyclosporine A (CsA) appeared in the scientific literature in several applications including vascularized bone and joint allotransplantation in rat, rabbit, and dog models^{11,35-42}. In 1982, Siliski described prolonged graft survival in vascularized whole-knee joint allografts in rabbits using cyclosporine A as the sole immunosuppressant^{41,42}. In 1987 Paskert et al. used Yaremchuk's vascularized knee allograft rat model, and reported the need for continuous CsA administration for long term allograft survival³⁹.

Based on these animal studies in 1994 Hoffmann et al. moved vascularized bone and joint allotransplantation into the clinical arena reporting successful vascularized allotransplantation of cadaveric human femoral diaphysis⁴³. Hoffmann et al. performed 8 clinical vascularized bone/joint allografts (3 femurs and 5 knees) using a CsA-based immunosuppressive regimen. In spite of promising early outcomes Hoffmann et al reported immunosuppression-related complications in five of the eight patients. These included allograft rejection, infection and/or thrombosis of the graft's vascular pedicle. These complications were reported to have led to bone instability, impaired healing and in some cases the need to remove the allografts. For example, one of the patients who received a knee joint allograft experienced a fatigue fracture of the tibial plateau, which eventually led to removal of the allograft. In this case it remained unclear to the authors whether the resulting bone instability and/or altered healing were caused by the rejection process or by a direct effect of the immunosuppressive drug on the bone¹⁷.

In Hofmann et al.'s first clinical cases the immunosuppressant regimen used was a combination of CsA, Azathioprine (AZA) and prednisone. In other studies conducted in solid organ transplant recipients this same regimen was reported to cause post-transplantation bone loss leading to osteoporosis and fractures. This same regimen has been reported to cause post-transplantation bone loss leading to osteoporosis and fractures in older solid organ transplant recipients⁴⁴⁻⁴⁸. In mid to late 1990's several clinical kidney transplant studies demonstrated MMF to be superior to AZA in triple-immunotherapy regimens with CsA and corticosteroids⁴⁹⁻⁵¹. Furthermore, it

was shown that CsA/MMF combination therapy effectively prevented rejection of allografts containing bone (hindlimb allograft), while simultaneously minimizing drug-specific side-effects in a rat model⁵². Based on these reports, in the present study we used CsA/MMF/prednisone combination therapy in a pre-clinical swine osteomyocutaneous flap allotransplant model and measured bone quality and healing post transplant.

Early outcomes in these studies revealed excellent functional recovery with normal gait and weight bearing. These results were consistent with similar studies, in which rabbit knee joint allografts and canine vascularized bone and knee-joint allografts demonstrated early functional recovery, as assessed by weight bearing ability, gait, and range of active and passive movement, equivalent to that of autografts^{36,42}. These positive early outcomes were also reported by Hofmann et al. in their clinical cases. All but one patient, who's allograft was removed because of infection within the first post-operative week, were discharged from the hospital in four to eight weeks with partial (40 kg) weight bearing on two crutches¹⁷. These reports, along with our study indicate that cyclosporine-based immunotherapy does not seem to adversely affect early functional restoration of allografted bone or joints.

The healing rate at the unions between the donor allograft and recipient bones in animals receiving CsA/MMF/prednisone combination therapy has not been well defined in the literature. In a rat tibiofibula allograft model using long-term cyclosporin A monotherapy at dosages slightly lower than those we used (10 mg/kg/d) in our pig model, the rate of bone healing was similar. However, they observed that when present graft rejection caused delayed union⁸. Lee et al. reported evidence of periosteal callus formation by week 2 and bony union and remodeling by week 8 in a vascularized allograft rat knee hemi-joint transplant model receiving CsA monotherapy, 10 mg/kg/d³⁸. In a dog vascularized knee allograft model immunosuppressed with CsA and azathioprine Doi et al. reported good healing in 3/5 allografts as determined using bone scans, plain radiographs, and bone biopsies³⁶. Our radiographic findings were consistent with these studies as we observed normal bone healing at both proximal and distal radii unions at the end of our three-month period. In addition our histological examination of bone biopsies taken at the end of the study showed callus formation and no evidence of bony resorption, sclerosis or fracture. These findings are also consistent with the early follow up radiograph assessments reported by Hofmann et al. that showed callus formation and osseous consolidation of osteotomies but no evidence of fractures⁵³.

In addition to assessing the effects CsA/MMF/prednisone combination therapy has on bone healing in this study we also measured its effects on bone quality. The effects immunosuppressive therapy has on bone quality have been well documented in organ transplantation literature. In fact immunosuppressive therapy is known to be one of the leading contributing factors to post-transplantation osteoporosis. Especially long-term administration of corticosteroids is known to decrease bone density and increase the risk of bone fracture^{54,55}. In general, bone loss (decreased bone density) is most rapid during the first 12 to 18 months of therapy and is directly related to dose and duration of corticosteroid exposure^{56,57}. In the present study the dose of prednisone we used was 2.0 mg/kg/day on the first postoperative day and then tapered by 0.5 mg/kg/d every 3 days to a maintenance dose of 0.1 mg/kg/d after 1 month. Clinical studies have shown that prednisone dosages of 0.1 mg/kg/day or higher are associated with significant bone loss⁵⁶. However, studies also indicate that CsA, administered alone or in combination with corticosteroids may also contribute to bone loss. Animal studies on this topic report conflicting results. In vitro studies have shown CsA inhibiting bone resorption, which would suggest a protective effect against bone loss^{58,59} similarly some in vivo studies report that CsA causes a decrease in bone resorption and an increase in bone formation⁶⁰. Several other in vivo studies using CsA in rat femur allotransplant models report high turnover osteopenia and decreased bone density^{47,61-65} and coincide with our findings.

When interpreting our bone density and bone elastic coefficient data and comparing it to solid organ transplantation studies it is important to consider that the bone specimens we studied are allogeneic, and were subject to episodes of rejection. It is known from studies using non-vascularized bone allografts, that the recipient's immune response against a donor bone allograft causes bone loss⁶⁶. In fact our histopathologic evaluations showed signs of rejection in all bone specimens studied. Therefore, we were unable to distinguish between the effects of bone rejection versus the effects of CsA/MMF/prednisone immunosuppressant therapy on bone quality (bone density, acoustic velocity and elastic coefficient). This underlines the need to develop methods to detect early signs of bone rejection. If vascularized bone and joint allotransplantation is to become standard care this will be essential¹⁷.

In conclusion, at the dosage we studied, CsA/MMF/prednisone immunosuppressant therapy was not able to effectively prevent bone rejection. Bone density and elastic coefficient of the allografted bone was significantly reduced three months post-transplant. The immunosuppressive drug com-

ination used in this study and the occurrence of rejection episodes do not appear to impair physiologic bone healing as evidenced by early and solid bony union of allografts. These findings could have important implications for the long-term outcome of vascularized bone and joint allotransplants. For vascularized bone allotransplantation to become standard care it will be essential to develop alternatives to immunosuppressive drugs or new drugs that effectively prevent rejection with minimal toxic side effects on bone quality.

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Chapter 2

Bone quality in swine composite tissue allografts: effects of combination immunotherapy

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INTRODUCTION

The prevalence of patients in the US with limb loss, excluding loss of fingertips or toes, is estimated to be 1,235,000; with an annual incidence of 50,000 new amputations¹. Until recently only three treatment options were available to individuals with limb amputations: reattachment, reconstruction with autologous tissues or replacement with prosthetic devices. The first of these options, reattachment, typically gives the best outcomes because the defect is reconstructed using like tissues. However, more often than not, the amputated limb cannot be salvaged and reattached, either because the trauma/disease causing the loss destroys the limb beyond use (major crush injuries, severe burns, invaded by tumor) or because the limb(s) never existed in the first place (congenital birth defects). In these cases the alternative treatment of autologous tissue transfer usually requires many revision surgeries and years of rehabilitation. Compared to reattachment, the functional and aesthetic outcomes are poor² and prosthetic devices only provide limited functional return.

Recently a new clinical treatment option became available for limb amputation: composite tissue allotransplantation (CTA), in which an amputated limb is replaced by the limb of a brain-dead, heartbeating donor³. The concept of limb transplantation precedes solid organ transplantation by centuries⁴ and yet although the latter has become standard care, CTA has remained largely experimental, not entering the clinical arena. The main reason that CTA has not advanced into the clinical setting is that until recently the immunosuppressive drugs available clinically were not capable of preventing skin rejection when administered at safe doses. In 1997, our laboratory conducted a series of experiments in a preclinical pig forelimb CTA model and demonstrated that the new combination immunosuppressive therapy tacrolimus (FK506)/mycophenolate mofetil (MMF)/prednisone, being used at the time in clinical organ transplants, effectively prevented rejection while causing minimal systemic toxicity⁵. From September 1999 to July 2003, 18 patients received 24 hand transplants worldwide (12 unilateral, 6 bilateral) using FK506/MMF/prednisone immunosuppressive therapy with 100% graft survival at 2 years follow up. Two graft failures were due to noncompliance to the treatment in one case and severe skin inflammation in the other³.

In spite of this early success, little is known about the effect FK506/MMF/prednisone immunosuppression therapy has on bone quality and healing in a CTA. Organ transplantation literature describes decreased bone quality (loss of bone mass) as one of the specific side effects of immunosuppressive therapy^{6,7}. However, the effects of an FK506/MMF/prednisone com-

bination regimen have not been described. In CTA procedures where the bone component is essential for long-term functional outcomes (e.g. hand, knee, femur), bone quality and healing is central to the success of the procedure⁸. With this in mind, we designed the present study to assess the effect FK506/MMF/prednisone immunotherapy has on bone quality and healing in a preclinical swine forelimb CTA model.

MATERIALS AND METHODS

In nine outbred pigs (13-24 kg) radial forelimb CTA flaps were transplanted from size-matched donor animals. The recipient animals received oral FK506/MMF/prednisone combination immunotherapy for 3 months. Allograft rejection was assessed by daily visual inspection of the flap's skin and histopathologic examination of skin biopsy specimens. Bone quality was studied pre- and posttransplant by measuring acoustic velocity and bone density of a portion of the radius. In two control pigs not receiving immunosuppression, autografts were performed using the same radial forelimb osteomyocutaneous free flap. Bone quality in these animals was only performed at 3 months posttransplant because no bone specimens were available for pretransplant measurements. Bone healing was assessed using radiographic analysis.

Animal care: Twenty age-matched (6-8 weeks old) and size-matched (13-24 kg) outbred farm pigs were used in this study. Donor and recipient animals were purchased from different suppliers to assure MHC mismatch. Animals were housed in separate cages in light (12hr-12hr), temperature (22°C) and airflow-controlled rooms. Animals were fed standard diets and were provided with water ad libitum. After an initial physical examination, baseline laboratory tests were performed (complete blood cell count with differential, electrolyte levels, and liver function tests) to assess each animal's general health. Pretransplant crossmatching was performed for each donor-recipient pair to assure MHC disparity and avoid hyperacute rejection. At the end of the 90-day experimental protocol animals were euthanized with 6ml of beuthanasia[®] (Schering-Plough Animal Health Corp., Kenilworth, NJ). This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, Publication No. [NIH] 86-23).

Pig forelimb CTA model: Orthotopic allotransplantation of right radial forelimb CTA flaps were performed as described in detail elsewhere⁹. Briefly, flaps were based on the brachial artery and cephalic vein and consisted of the flexor carpi radialis muscle, a segment of the median nerve including its branch to the transplanted muscle, a segment of the radius, and an island of overlying skin extending from the craniomedial to the cranio-lateral aspect of the right forelimb. After skin closure and application of a wound dressing, a fiberglass cast was applied to the forelimb. A window was created in the cast over the skin portion of the flap to permit daily inspection and regular biopsies of the graft until the cast was removed 3 weeks post-transplant.

Experimental groups: Nine CTA and 2 autografts operations were performed using 20 animals. All CTA flap recipients received once-daily oral FK506/MMF/prednisone combination therapy. FK506 (Prograf 5-mg capsules, Fujisawa USA, Deerfield, Ill) 1.5 mg/kg/d was begun on the morning of surgery, with the dose subsequently adjusted to maintain 24-hour whole-blood trough levels between 3 and 8 ng/ml by the Incstar ProTrac II enzyme-linked immunosorbent assay. MMF 500 mg per day was begun on the morning of operation. Methylprednisolone 500 mg was administered intravenously during the procedure just before flap transplantation. Prednisone 2.0 mg/kg/d was begun on the first postoperative day and then tapered by 0.5 mg/kg/d every 3 days to a maintenance dose of 0.1 mg/kg/d after 1 month. Animals in the treatment group did not have access to food during the night to increase the likelihood of their ingesting and absorption of the immunosuppressive drugs, which were mixed with small amounts of food in the morning. Finally, drug doses were maintained constant and were not adjusted according to clinical signs of rejection. The operations in the autograft controls, consisted of elevating two radial forelimb osteomyocutaneous free flaps and reimplanting them to the same location in the same animals.

Postoperative care: For infection prophylaxis, procaine penicillin G, 30,000 units/kg was administered intramuscularly to all recipient pigs for 10 days. Complete blood cell counts with differentials, electrolyte levels, and liver function tests were determined 3 times per week for the first week and weekly thereafter until the end of the study. Twenty-four hour FK506 trough levels were determined daily for the first 3 weeks, 3 times per week for the next 3 weeks, and then weekly thereafter. All recipients were followed up for 3 months for the occurrence of acute rejection, graft loss, or

death. Rejection was assessed clinically by daily visual inspection of the flap skin by two examiners. Skin biopsies were performed on days 0, 2, 4, 7, 10, 14, 21, 30, 45, 60, and 90. The skin biopsy specimens were initially fixed in 10% buffered formaldehyde and then transferred to and stored in 70% ethyl alcohol. For analysis the tissue sections were stained with Hematoxylin and Eosin stains. Visual and histologic scoring systems that were formulated in our previous work with rejected CTAs in pigs, to grade the severity of rejection were applied to the current study¹⁰. Skin color, presence or absence of blister formation and extent of bleeding from biopsy site were the relevant parameters selected for visual scoring. The severity of vasculitis, folliculitis, dermal inflammation, and epidermal degeneration was used for histologic scoring, with an overall grade assigned to each: 0= none, I= mild, II= mild to moderate, III= moderate and IV= severe.

Graft rejection, defined as severe flap cyanosis and sloughing (indicating complete rejection), animal death, or the end of the 3-month follow-up period were considered the study endpoints. At this study endpoint complete necropsy was performed on all animals.

Bone quality measurements: Bone quality was evaluated by calculating the bone biomechanical elastic properties with data obtained from acoustic velocity and bone density measurements of our radius bone grafts^{11,12}. In the CTA group, three segments of bones were collected for analysis: 1) radial bone segments removed from recipients to make way for the donor CTAs were used for "normal" bone measurements, 2) radial bone donor CTA segments harvested from the recipients at the end of the study were used for posttransplant CTA bone measurements, and 3) radial bone recipient segments taken from the contralateral limbs of the CTA animals at the end of the study were used to determine the effect of immunosuppression on intact bone. In the autograft controls, the radial bone segments were removed and immediately replaced; therefore, no bone sample was taken for pretransplant measurements. Thus in the autograft group, only radial bone segments harvested at the end of the study were available to perform posttransplant measurements in autograft controls. In this group, radial bone segments were divided into four segments (total of eight) to generate a representative mean density and acoustic velocity. The acoustic velocity was measured using a longitudinal ultrasound transmission technique and bone density was measured using Archimedes' principle¹³. Bone samples previously stored at -70°C were thawed immediately before testing. Each of the respective radius bones was cut perpendicular to their long

axis using a low speed diamond saw (Beuler Isomet; Lake Bluff, IL). A second cut parallel to and approximately 1 cm from the first cut was made. For measurement of the acoustic velocity each specimen was placed between an ultrasound transmitting and receiving transducer (Panametrics, SmH2; Waltham, MA). With the cortical bone pathway to be measured centered on the transducer surfaces, a 15 V square wave signal was applied to the transmitting transducer using a function generator (Model 3011, BC Precision; Chicago, IL). This input signal was also connected to one channel of a digitizing oscilloscope (model 54501A, Hewlett Packard; San Jose, CA). The output from the receiving transducer was connected to a second channel of the oscilloscope. By comparing the onset of the input and received signals, the transmission time, Δt , of the ultrasound energy crossing through the specimen could be determined. The length of the bone specimen (l) was measured using a micrometer. The acoustic velocity (v) was then calculated as follows: $v = l/\Delta t$, where l is the length of the specimen spanning the ultrasound transducers. All the measurements were carried out at room temperature (20°C). Results of acoustic velocity measurements were expressed in GPa.

Bone density measurements were obtained according to Archimedes' principle. The mid-diaphyseal cortical bone specimens previously used in the acoustic velocity measurements were stored in distilled water in a 360 mm Hg vacuum for 30 minutes. Next, the hydrated specimen was weighed both in and out of the water bath. Bone density measurements were calculated as follows: density (ρ) = $(A/A-B) \times P$, where A is the weight of the hydrated bone, B is the weight of the hydrated bone submerged in water, and P is the density of distilled water at a given temperature. $A-B$ is the equivalent to the volume of the bone specimen. Results of bone density measurements were expressed in kg/m^3 .

To further assess the potential differences in bone quality of the radius specimens pre and posttransplant, a longitudinal elastic coefficient (E) was calculated for each bone specimen using the relationship: $E = \rho \times v^2$, where ρ is the radius density and v the measured acoustic velocity¹². Assuming that the acoustic wave pathway in bone is homogeneous, the elastic coefficient represents the intrinsic longitudinal stiffness of the specimens. Although no clear quantitative relationship exists between the elastic modulus of bone and its absolute mechanical strength^{13,14}, recent studies show a relatively strong correlation between its modulus and ultimate strength¹⁵.

Bone healing measurements: Bone healing was assessed using serial radiographic and histopathologic measurements. After adequate sedation,

anterioposterior and lateral radiographs were taken of the transplanted forelimb twice during the first week, monthly thereafter and prior to sacrifice with standard 11 x 14 inch film-plates at a 40 inch film-to-tube distance at 50 kV and 4mAs (OEC -Diasonic X-ray Imaging Systems; Salt Lake City, UT). Radiographs were graded by the modified radiologic Weiland et al. scoring system in Table 1⁶.

Table 1. Modified Weiland scoring system for radiographs.

Grade	Proximal junction/ Distal junction	Body of the graft
0	Complete resorption/ pseudarthrosis	Complete resorption/ pseudarthrosis
1	Severe resorption	Severe resorption
2	Mild resorption	Mild resorption
3	Resembling postoperative	Resembling postoperative
4	Early union	Mild new (+2mm)
5	Solid union	Moderate new bone (+4mm)
6	Beginning remodeling	Beginning remodeling

Statistical analysis: Data are reported as means and standard deviation (SD). Significant differences between groups were detected using paired t-tests. Differences were considered significant at $p < 0.05$.

RESULTS

CTA model: None of the radial forelimb flaps failed as a result of technical problems associated with free tissue transfer and there was no morbidity attributed to the transplant procedure. Four animals did not survive until the 3-month endpoint of the study: Three died from pneumonia on days 29, 30, and 83 without signs of limb rejection. Of these 3 pigs, the one that died on day 83 was considered to be far enough out and close enough to the 90-day study endpoint to permit bone analysis studies so this animal's bone was included in the measurements. The fourth pig died on day 42 from gastric rupture at which time histologic evidence of mild CTA flap rejection was found. In the experimental "CTA" group (n=9) none of the forelimb CTA flaps was lost due to rejection. Of the six animals whose flaps

were included in the bone measurements, three flaps developed signs of mild (grade I-III) rejection during the first postoperative week as assessed by visual inspection and histopathologic examination of the flap's skin. All of these rejection episodes resolved spontaneously without adjustment of the drug dosage. The two autograft control animals survived without complications to the 3-month endpoint of the experiment. Serum electrolytes, liver function tests, white blood cell and platelet counts remained within normal limits in all animals throughout the study except during times of infection.

Bone quality: The results of the bone density, velocity and the calculated elastic coefficient are summarized in Table 2 and Figures 1 and 2. The mean bone density of normal bone, CTA bone, and CTA-contralateral limb bone was not statistically different (Table 2). However, the mean bone density of autograft bone was significantly different from CTA-contralateral bone ($1560.1 \pm 137.4 \text{ kg/m}^3$ vs. $1787.0 \pm 171.0 \text{ kg/m}^3$, $P=0.0182$). There were significant differences in the mean acoustic velocity between normal bone and both CTA bone and autograft bone (Table 2). However, no significant differences in acoustic velocity were found between autograft bone and CTA bone.

Table 2. Result summary of bone density and acoustic velocity measurements (mean \pm SD).

Bone Sample	Density (kg/m³)	Acoustic Velocity (m/s)
Autograft (n=2 x 4)	1560.1 \pm 137.4 ^a	2939.0 \pm 218.4 ^b
CTA (n=6)	1599.4 \pm 141.4	3060.3 \pm 380.7 ^c
Normal (n=6)	1677.5 \pm 80.8	3628.1 \pm 87.6 ^d
CTA-contralateral	1787.0 \pm 171.0	3806.0 \pm 173.7

^ap= 0.0182 vs. CTA-contralateral

^bp= 0.0011 vs. Normal and p= 0.0011 vs. CTA-contralateral

^cp= 0.0206 vs. Normal and p= 0.0094 vs. CTA-contralateral

^dp= 0.0233 vs. CTA-contralateral

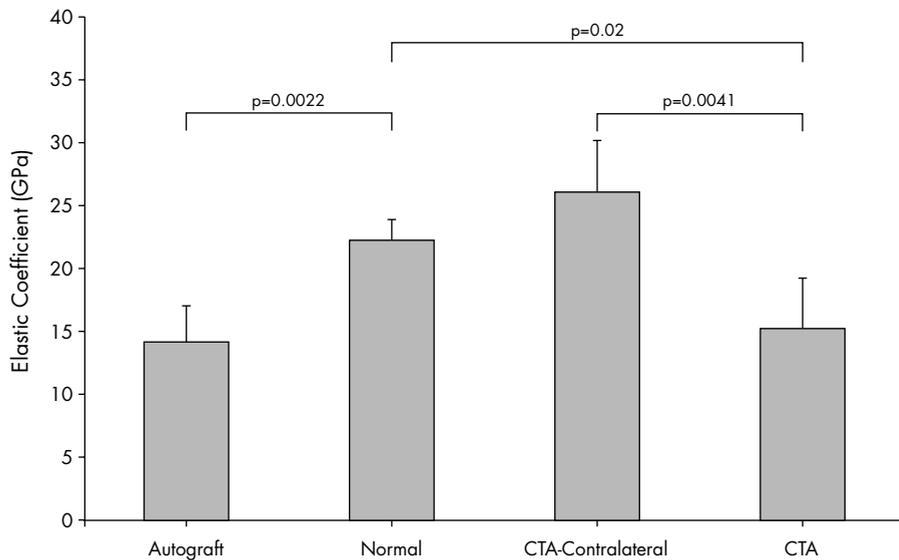


Figure 1. Comparison of elastic coefficient results (mean \pm SD) obtained from CTA, autograft, normal and CTA-contralateral bone samples.

The mean elastic coefficient of normal bone was significantly different from both CTA bone and autograft bone (Table 2). When the mean elastic coefficient was compared between CTA-contralateral limb bone and CTA bone, a significant difference was found (Fig 1). To determine whether the FK506/MMF/prednisone immunotherapy had an effect on bone, we first compared autograft bone with CTA bone to determine whether the two groups behaved differently, and we found that the two were statistically similar (Fig 2). We then proceeded to compare the mean elastic coefficient of normal bone with bone from CTA-contralateral limbs to determine whether immunotherapy had an adverse effect on intact bone, and we also found that there was no significant difference between the two (Fig 2).

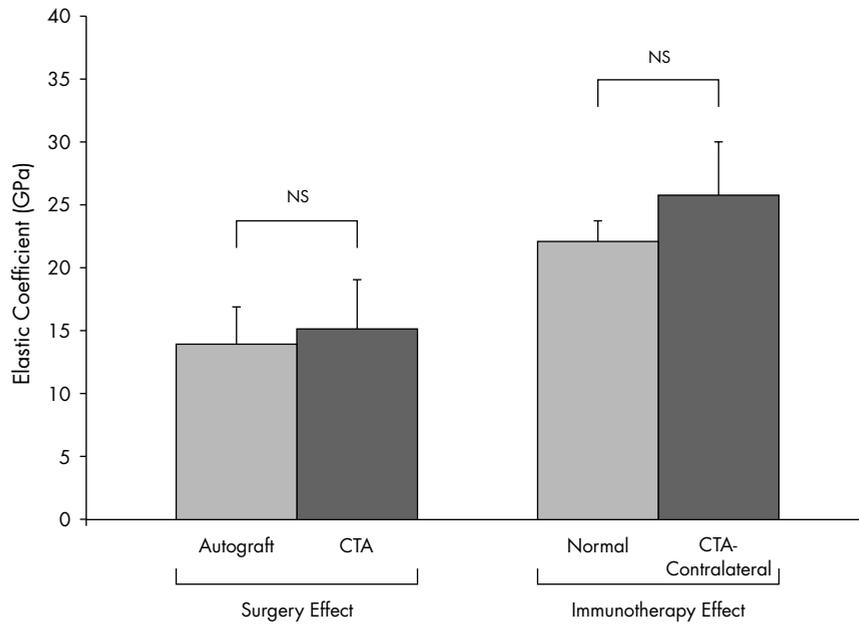


Figure 2. Comparison of elastic coefficient results (mean \pm SD) showing the effect of surgery and immunosuppression on operated (autograft vs. CTA) and intact bone (normal vs. CTA-contralateral), respectively.

Bone healing: The radiographic results of the transplanted forelimbs depicted in Table 3 show that the proximal and distal junctions of the transplanted radii were healing with evidence of solid bony union and the beginning of graft remodeling. There was no radiologic evidence of bone resorption or pseudoarthrosis present in any of the animals (Fig 3). These results were supported at necropsy with evidence of callus formation at the proximal and distal junction of the transplanted radii.

Table 3. Results of radiological findings of radial forelimb free flap in the CTA bone (n=6) and in autograft bone (n=2). Radiographs were scored using the Modified Weiland scoring system.

Pig No.	Proximal junction	Distal junction	Body of graft
1	5	5	5
2	6	6	5
3	5	5	5
4	6	6	6
5	5	5	6
6	6	6	5
Control 1	6	6	6
Control 2	6	6	6

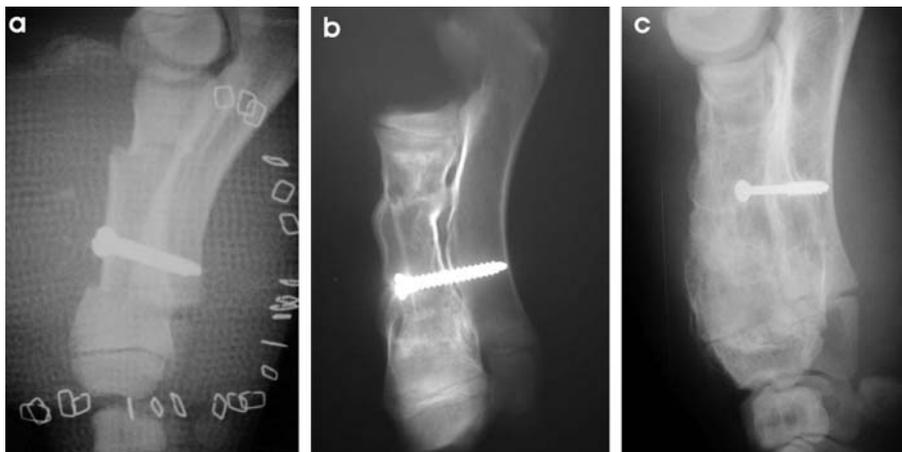


Figure 3. Post-op radiographs of transplanted radii in CTAs. a. Immediate post-op radiograph of pig forelimb with fiberglass cast. Note nonunion of proximal and distal regions of the bone graft. b. Three month post-op radiograph of immunosuppressed pig forelimb with remodeling of the radius and union of proximal and distal graft regions. c. Autograft (control) pig forelimb three months post-op with remodeling of the radius and union of proximal and distal graft regions.

DISCUSSION

In composite tissue allotransplants containing bone, optimal bone quality and healing are essential for successful long-term functional outcomes.

Unfortunately, many of the immunosuppression drug regimens used to prevent rejection in allotransplant procedures are known to have detrimental effects on bone⁷.

The experiments described here focus on the effects FK506/MMF/prednisone combination therapy has on bone quality and healing. Therefore we will limit the scope of this discussion to this drug regimen.

Recently, FK506/MMF/prednisone, a drug regimen widely used in kidney¹⁷, liver¹⁸ and kidney-pancreas transplants¹⁹ has also been shown to effectively prevent rejection in composite tissue allotransplants both in animal models and in human hand transplants^{5,20}. In spite of this success, to our knowledge, the effect this drug regimen has on bone quality and healing has not been reported.

In clinical studies the use of FK506 in combination with corticosteroids has been shown to be an important factor in posttransplantation bone disease²¹. However, in these clinical studies the effects of FK506 alone were difficult to characterize. In studies performed in a rat model FK506, administered as monotherapy²², was also reported to decrease bone mass. In these studies FK506 was administered at a relatively high dose (5 mg/kg/day). In contrast, Inoue et al. showed that FK506 administered at a lower dose (1 mg/kg/day) did not influence bone mass as measured by bone density measurements²³. In the present study in our pig CTA model we used low dose FK506. Dosages were adjusted to maintain 24-hour whole-blood trough levels between 3 and 8 ng/ml and never exceeded the dose of 1.5 mg/kg/day.

In another study by Dissanayake et al. in a rat model receiving MMF (30 mg/kg/day), bone mineral metabolism and bone volume were measured and found to be unaffected even though bone osteoblastic activity was reported to be decreased²⁴.

Of all the immunosuppressant drugs reported corticosteroids have been described to have the greatest deleterious effect on bone. The mechanism by which they weaken the bone is by upsetting the normal bone remodeling process. Corticosteroids have been shown to accelerate bone resorption (bone loss) while decreasing bone formation. This disruption of normal bone homeostasis leads to reduced bone density and increased risk of bone fracture. In general, the increase in bone loss is directly related to the dose and duration of corticosteroid exposure and is most pronounced during the first 12 to 18 months of therapy²⁵. In this study the dose of prednisone we used was 2.0 mg/kg/day on the first postoperative day and then tapered by 0.5 mg/kg/d every 3 days to a maintenance dose of 0.1 mg/kg/d after 1 month. Clinical studies showed that nearly all persons treated

with prednisone dosages of 0.1 mg/kg/day or higher experience significant bone loss²⁵. Perhaps the high doses of prednisone used during the early postoperative period could be avoided by treating CTA recipients with OKT3 or other monoclonal antibodies. OKT3 therapy blocks human CD3 molecule on T cells and has been effectively used in the treatment of steroid-resistant rejection in solid organ transplantation^{26,27}. However the risks and benefits of antibody therapy need to be carefully considered, particularly for CTA procedures²⁸. In this study we found a slight but not significant reduction of the mean bone density posttransplant. Interestingly, the two animals in which posttransplant bone density was not reduced had the highest FK506 trough levels of all six pigs (pigs 2 and 5). Although we did not observe a significant reduction in bone density in this study, we did find an important reduction in the elastic coefficient posttransplant in all six animals (CTA), indicating that bone quality was significantly reduced in our pig forelimb CTA model. This led us to the question: was the observed reduction in the elastic coefficient caused by the surgery or by the immunosuppression? We compared autograft bone (surgery without immunosuppression) with CTA bone (surgery with immunosuppression) and found that the elastic coefficient was reduced similarly in both groups, suggesting that the operation was the cause (Fig 2). To further confirm this finding, we compared the elastic coefficient of normal bone (without surgery and without immunosuppression) with CTA-contralateral limb bone (without surgery and with immunosuppression) and found that the elastic coefficient was similar in both groups (Fig 2), suggesting that FK506/MMF/prednisone combination therapy administered for 90 days did not reduce bone quality. When interpreting these findings, one must consider the relatively short duration of the study as well as the young age of the animals used, both of which could influence these results. In the clinical setting, CTA recipients will most likely be beyond an age of rapid bone growth and will receive immunosuppression for life. These factors could contribute to their experiencing negative effects on bone quality that are not evidenced by the findings in this study⁷. At present, patients receiving clinical CTA procedures require lifelong immunosuppression therapy. Unfortunately the drug regimen shown to be effective in preventing CTA rejection, FK506/MMF/prednisone combination therapy, contains a steroid known to reduce bone quality⁷. In order to minimize the risk imposed by this drawback, one must consider adding monitoring of bone quality to these patients' regular checkups. In the present study, we assessed bone quality by measuring acoustic velocity and bone density using an ultrasound transmission technique and Archimedes principal, respectively. Quantitative ultrasound methods that

utilize a predictive value of acoustic velocity have been developed and introduced in recent years for assessing skeletal status^{29,30}. More recently, several of these devices have been introduced into the clinical arena and provide a method for noninvasive assessment of bone pathologies such as osteoporosis and fracture at peripheral skeletal sites³¹. The attractiveness of these devices lies in their low cost, portability, ease of use, and perhaps most importantly the fact that patients are not exposed to ionizing radiation, making these tests noninvasive. This latter feature could be particularly valuable for bone-containing CTA recipients who require lifelong immunosuppression and therefore lifelong monitoring of bone quality. In these patients, it is advisable to start measuring the bone status in an early stage after transplantation, to start treatment with therapy to increase bone quality and maintain the functional integrity of the CTA if necessary.

In addition to assessing bone quality in this study we also measured the effects FK506/MMF/prednisone combination therapy has on bone healing. Using radiographic and histologic analysis we found that this drug regimen did not alter normal bone healing in our CTA model. All transplanted CTA flaps displayed active bone remodeling and evidence of bony union at both the proximal and distal junction of the transplanted radii at 3 months. In contrast to our findings other investigators have found in a rabbit model that corticosteroids administered at doses similar to those we used slow fracture healing³². Our radiographic data suggest that early bony incorporation of the graft by the host occurs by month 3 in our pig CTA model receiving FK506/MMF/prednisone combination therapy. These results were supported at necropsy with evidence of callus formation at the graft-host junction.

In conclusion, although FK506/MMF/prednisone combination therapy provides durable rejection-free CTA survival, it also permits the physiologic healing of bone to proceed as evidenced by early and solid bony union of the allografts. However, when looking at the biomechanical elastic properties of the bone component in our CTA flap we found a significant decrease in the elastic coefficient post-transplant, which appears to be induced by the transplant procedure itself more than the immunosuppression regimen. These results should be taken into account when performing clinical CTA procedures in which bone is one of the transplanted tissues, such as hand transplantation. The bone and mineral status of these patients should receive special attention both pre and post-transplant to avoid critical bone loss with a risk of fracture and thereby failure of the CTA.

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Chapter 3

Low-dose immunosuppression in a rat hind-limb transplantation model

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INTRODUCTION

Transplantation of composite tissue allografts (CTAs) from cadaveric donors offers an excellent alternative to conventional reconstructive methods for repairing large tissue defects resulting from traumatic injury, tumor extirpation and congenital birth defects. In spite of its promising potential composite tissue allotransplantation has not been widely applied in the clinical setting due primarily to the toxicity associated with the immunosuppressive drugs needed to prevent graft rejection in these procedures.

This toxicity is not necessarily due the immunosuppressive drugs per se but rather to the high doses required to prevent rejection and ensure long-term survival of the highly immunogenic skin component of CTA. The risks associated with high dose immunosuppression together with the fact that CTA procedures would be used to treat non-life-threatening tissue defects has raised the question "are the risks worth the benefits of these new procedures?" This risk versus benefit debate is perhaps the primary reason why this promising new reconstructive procedure has not gained widespread clinical application.

The ultimate goal of transplantation research is to replace toxic immunosuppressive drugs with a method of inducing transplantation tolerance³³. Until transplantation tolerance becomes a clinical reality, reducing the toxicity of current immunosuppressive regimens is an approach worth pursuing. One such approach is the use of combination immunosuppression therapy, which allows lower doses of individual drug to be used and thus causes less toxicity¹⁸.

In animal composite tissue allotransplantation studies different combinations of immunosuppressive drugs have been used with varying success. Using a rat hind limb transplant model, various investigators reported that combinations of tacrolimus and rapamycin¹⁵ or tacrolimus and deoxyspergualin (DSG)³⁰, prolonged CTA survival. Benhaim et al. demonstrated indefinite limb survival in a fully mismatched rodent model using combination therapy with cyclosporine A (CsA, 1.5 mg/kg/day) and mycophenolate mofetil (MMF, 15 mg/kg/day)⁷. However, at these low doses the investigators still reported episodes of rejection in 11% of their animals⁷. Based on the fact that tacrolimus has been demonstrated to have 100 times the immunosuppressive effect of CsA at equivalent doses^{12,4}, it could be expected that substituting tacrolimus for CsA in the above-mentioned study could provide improved survival of CTAs in a similar model.

The purpose of the present study was to determine in a rat hind limb CTA model whether low dose tacrolimus administered in combination with MMF prevented rejection and minimized toxic side effects.

MATERIALS AND METHODS

Wistar Furth (WF, RT1A^u) rats received hind limbs transplanted from ACI (RT1A^b) donor rats and were allocated into one of three groups: group I (syngeneic), group II (allogeneic, not treated) and group III (allogeneic, treated with tacrolimus and MMF combination immunotherapy). Rat limb rejection was assessed daily by visual inspection and by scheduled skin and muscle biopsies. At the end of the study, a histopathological exam was also performed on all tissues. Flow cytometry analysis was performed to detect the presence of donor chimerism and mixed lymphocyte reaction (MLR) for in vitro assessment of tolerance.

Animal care: Animals were kept in separate cages in temperature-controlled (24°C), light-regulated (12 h/day), and air flow regulated rooms. They were provided with a balanced rodent diet and water ad libitum. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for all surgical procedures, and sterile techniques were used for all surgery. Upon completion of the experiments, rats were killed with an overdose of sodium pentobarbital. The study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86-23).

Animal model: Strong major histocompatibility complex (MHC) mismatch male rats (weighting 200g–250g) were used in this study. ACI rats (RT1 A^b), as donors and Wistar Furth rats (WF, RT1 A^u), as recipients were purchased from Harlan Sprague Dawley (Indianapolis, IN). Twenty rats were used in this study and were allocated in three groups: group I (n=4) WF rats received syngeneic hind limbs from naïve WF rats; group II (n=6), WF rats received allogeneic hind limbs from naïve ACI rats without immunosuppression regimen; group III (n=10), WF rats received allogeneic hind limbs from ACI rats, and were treated with tacrolimus and MMF.

Donor surgery: A circumferential skin incision was made just proximal to the mid-thigh area. The femoral artery, vein and nerve were dissected, and the individual muscle groups of the hind limb were identified and divided as proximally as possible to their tendinous origins. Care was taken to not injure the profunda femoris vein. The sciatic nerve was identified and divided. The femur was exposed and divided transversely at the mid-shaft using a hand saw. The donor rat was then given the anticoagulant heparin (50 U), (Elkins-Sinn, Inc. Cherry Hill, NJ), which was injected intravenously into the opposite femoral vein. After 10 min, the femoral artery was clamped as proximally as possible and cannulated with a 24-gauge catheter. The limb was flushed with a solution of heparinized Ringer's Lactate (1 U of heparin per 1 ml of Ringer's solution) through the cannulated artery. Vascular flushing was maintained for 10 min until the backflow from the vein was observed to be clear. The femoral vein was ligated and sectioned, as proximally as possible. The dissected limb was isolated and immediately placed in cold Ringer's lactate, ready for transplantation.

Recipient surgery: The operative procedure to remove the native recipient limb was similar to that performed in the donor, except that the recipient was not given heparin, and all the neurovascular structures were cut as distally as possible to allow for maximum length during the anastomosis of the new limb. The bone was fixed using a 2 mm Kirschner wire (~1.5 cm in length) inserted intramedullary. The femoral vessels and the nerves were anastomosed using microsurgical technique (10-0 Nylon). The muscles and tendons were approximated using interrupted suture (5-0 Nylon), and the skin was closed using interrupted absorbable suture (5-0 Vicryl). The recipient rat was then returned to its cage where it was allowed to recover from anesthesia. For pain relief, ketoprofen (3-5 mg/kg: i.m.) was administered twice a day over the first three days and thereafter as needed if animals displayed signs of distress. A solution (Butler® bitter safe mist, Columbus, Ohio) was sprayed daily (three times) onto the transplant area to prevent automutilation (chewing) of the insensitive, transplanted limb for the first 8 weeks.

Visual assessment of rejection: The transplanted limb was observed daily for signs of rejection (edema, change of color and necrosis of the skin) and for patency of vessels. Previously described visual scoring criteria were used for the assessment of graft rejection³⁹. Time of rejection was defined as the day when either, the softened surface of skin could be wiped

away with the gentlest touch or when the entire surface was hard and scarified with hair loss.

Histopathology: Using a 2-mm biopsy punch, skin and muscle biopsies from the transplanted limbs were taken at 14 days and monthly after transplantation. Biopsies in Group II were taken every two days until frank rejection was present. All animals were followed-up for 5 months or until the limb was rejected. Tissues from skin, muscle, spleen, lymph nodes, small bowel, lung, liver, tongue, thymus, bone and bone marrow were harvested, fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin and eosin for microscopic examination. A pathologist read all the slides in a blinded fashion, and scored the histological sections based on an established grading scale¹⁰.

Peripheral blood assays: Five-hundred microliters of blood were collected in 2 separate vials (EDTA and heparin) for biochemical analysis of blood (CBC, electrolyte and liver profiles) at the time of killing. The PRO-Trac II Tacrolimus Elisa kit (DiaSorin), and Date EMIT assay kit, were used to measure peripheral blood levels of tacrolimus and MMF, respectively.

Flow cytometry: Fluorescence-activated cell sorter (FACS) analysis was performed after limb transplantation for detection of the levels of donor chimerism. Briefly, peripheral blood from rats was collected in heparinized plastic vials and aliquots of 100 μ l were stained with purified anti-RT1A^u (NR3/31; rat IgG2a; Serotec) and biotinylated anti-RT1A^{ab} (C3; LOU/Cn IgG2b; Pharmingen) monoclonal antibody for 30 minutes. Using a similar procedure, the bone marrow cells (BMC) from femurs and tibiae in non-transplanted limbs, were flushed and analyzed for chimerism, by the use of flow cytometry at the time of killing.

Immunosuppressive treatment: The rats in group III were treated with a low-dose combination therapy that consisted of 1 mg/kg per day of tacrolimus diluted in 5% dextrose administered i.p. for 14 consecutive days, followed by 1 mg/kg twice a week thereafter, and of MMF powder (15mg/kg per day) that was reconstituted with saline solution and administered orally. During rejection episodes tacrolimus was administered daily for 7 consecutive days, and, thereafter the treatment was returned to the bi-weekly regimen.

Statistical analysis: All values are expressed as mean \pm SEM. Analysis of Variance (ANOVA) among groups was performed, and if statistical significance was found ($p < 0.05$) we performed a post-hoc unpaired t-tests to compare differences between two groups. In all experiments, animals survival times between groups were calculated and compared according to the Kaplan-Meier method.

RESULTS

Visual assessment of rejection: In group I, none of the rats showed any rejection signs, and they were killed at the end of the study at 5 months (Fig. 1).



Figure 1. Transplanted limb in a syngeneic WF animal 150 days after transplantation (group I). Note the healthy appearance of the transplanted limb with normal hair and nail growth. Limb function was normal except for toe contracture.

In all animals the postoperative edema disappeared after 7 days. In group II, the CTA limbs showed increasing edema postoperatively, up until the point when irreversible acute rejection was established. Skin coloration gradually changed from pinkish to reddish-purple, and finally to purplish-blue (Fig. 2).



Figure 2. Transplanted allogeneic limb without any treatment (ACI to WF recipients) 10 days after transplantation (group II). Rejection signs of severe edema with discoloration, formation of vesicles, and hardening of the skin are apparent.

The mean rejection time of limbs was 5.7 ± 1.5 days. In group III, postoperative edema of the transplanted limb disappeared completely after 10 days post-transplantation (Fig. 3) (see next page).

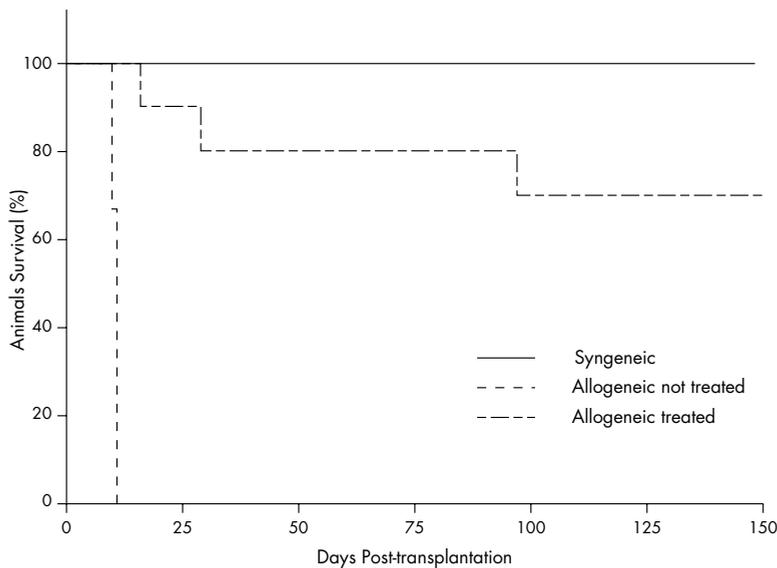


Figure 3. Percentage of animal survival between groups according to the Kaplan-Meier life-table method. In the syngeneic group, all animals completed the study (5 months). In the allogeneic group without treatment all animals rejected their limbs and were killed within 10 days of transplantation. In the allogeneic group with immunosuppressive drugs, seven of ten animals completed the study (5 months) and were killed.

Three of ten rats did not complete the study period. They either died or were killed prematurely at 16, 29, and 97 days after transplantation. The cause of death in the first rat (16 days) was not apparent; however, no rejection episodes were observed and no changes in immunosuppressive therapy were made. Automutilation (chewing) of the transplanted limb was the reason why the second rat was killed at 29 days post-transplantation, but no clinical or histological signs of rejection were observed. Only in the third animal (found dead at day 97 post-transplantation) was a rejection episode observed, and the dose of immunosuppressive drugs had to be adjusted. Seven of 10 rats survived for the length of the study and were killed at five months (Fig. 4). During the follow-up period, one rat had no rejection episodes, four rats had single rejection episodes, and two rats had multiple rejection episodes.



Figure 4. Transplanted limb from the immunosuppressed group (tacrolimus and MMF) 150 days after transplantation (group III). Note the healthy limb with normal black hair (from the donor ACI rat) and nail growth. Limb function was normal except for toe contracture.

Histopathology: In group I, none of the animals showed any signs of rejection during the study. Histopathologic analysis showed normal tissue architecture in all solid organs, as well as in muscle and skin from the CTA limb. Two rats presented marginal hyperplasia in the spleen, and in one of these rats a slight portal infiltration was also found. In group II, the findings from biopsies of skin and muscle from CTA limbs were consistent in all animals. Normal tissue architecture was seen at 2 days after transplantation, moderate rejection (increasing basal cell vacuolation and bulla formation in the epidermis) was observed at 4 and 6 days post-transplantation, and severe rejection (edema, vasculitis, complete necrosis and epidermal degeneration, and inflammatory infiltration in the dermis) was noted at 8 days post-transplantation. In group III, three of the ten animals did not complete the follow-up period, and at the time of death the transplanted limbs did not show any histological signs of rejection (Fig. 5).

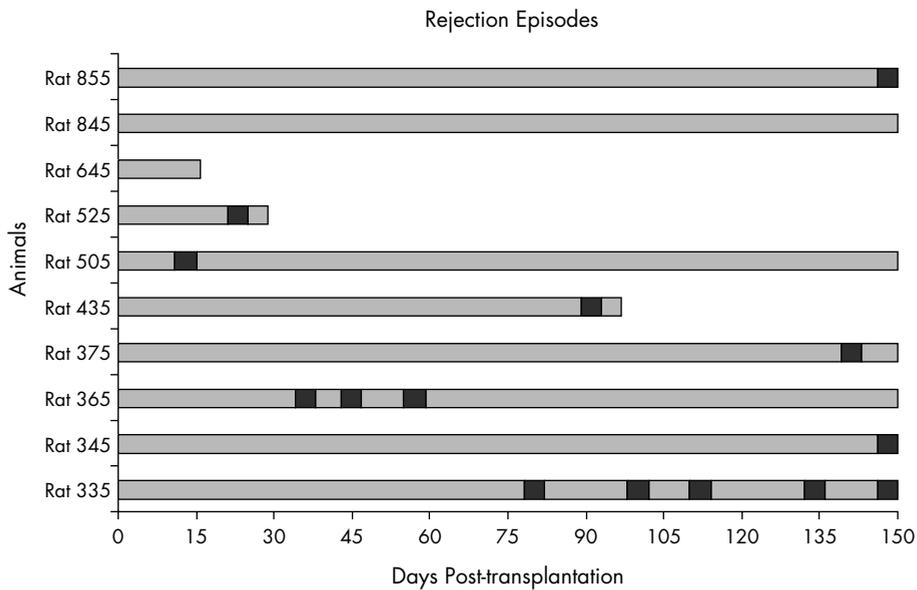


Figure 5. Number of rejection episodes (black bars) in the immunosuppressed group treated with tacrolimus and MMF (group III). Seven of the ten animals completed the study (5 months). At the time they were killed, four animals showed no clinical or histological signs of rejection of the transplanted limb.

However, in three of the seven remaining rats that did complete the study, their transplanted limb (at the time of killing) showed mononuclear dermal infiltration compatible with mild rejection (Fig. 6). With the rest of the animals (four rats, 57%) histology was normal, with no signs of rejection. In all seven animals the spleen showed a marginal zone of hyperplasia. The lymph nodes also showed hyperplasia (2 rats), atrophy (1 rat), congestion (1 rat), and normal architecture in the remaining 3 rats. The liver showed steatosis (2 rats), abscess (1 rat), abscess with ascending cholangitis (1 rat), and cellular infiltration (1 rat); in the remaining 2 rats the livers were normal. The histopathology of small bowel, lung and bone showed normal architecture in all the seven long-term follow-up animals.

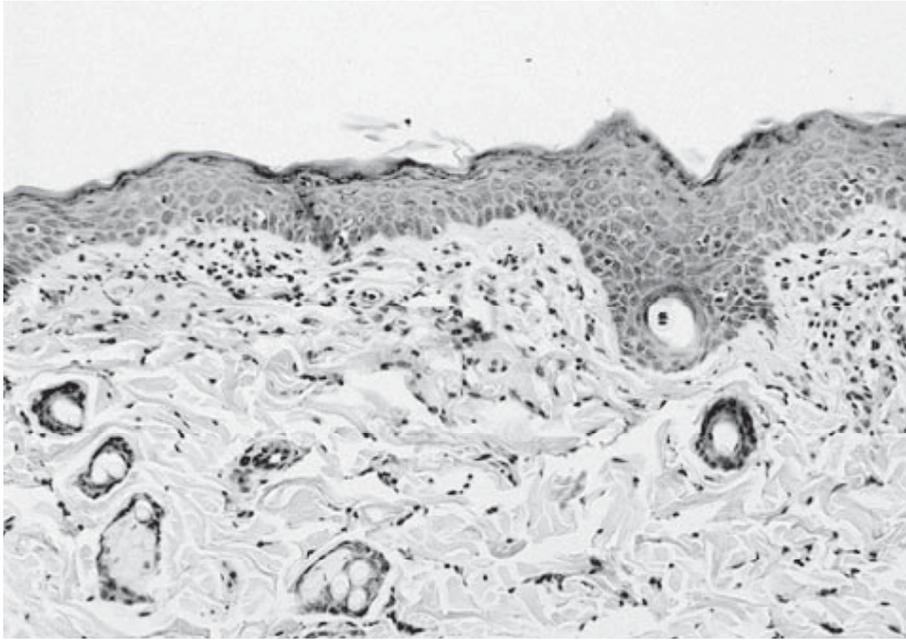


Figure 6. Histological section of donor ACI skin from the transplanted limb at the end of the study in an immunosuppressed animal (group III). This animal did not have any rejection episodes during follow-up. At the time it was killed, skin sections showed mild lymphocyte infiltration. (H&E, 400X)

FACS analysis: Only in group III was FACS performed on peripheral blood lymphocytes (PBLs) (at 30, 60, 90 and 150 days after transplantation) and from BMCs in the opposite limbs (at the time of killing) to assess the presence of donor chimerism. The levels of donor chimerism in PBLs ranged between 0.5 and 25 %. Similar levels were found in BMCs from the host's non-transplanted limbs.

Blood analysis: Blood analysis was performed at the end of the study in two animals in the immunosuppressed group. The analysis showed 30% and 35% of hematocrit (values comparable with the syngeneic group). White blood cells were $4 \times 10^3/\mu\text{l}$ and $6 \times 10^3/\mu\text{l}$. Platelet counts were $320 \times 10^3/\mu\text{l}$ and $540 \times 10^3/\mu\text{l}$, and glucose levels were 165 and 200 mg/dl. Drugs levels in these two animals were 8.9 and 11.5 ng/ml (for tacrolimus) and 1.6 and 3.8 $\mu\text{g/ml}$ (for MMF).

DISCUSSION

Over the past decade, the concept of administering low doses of different immunosuppressive drugs, each, acting via different mechanisms, to deliver potent immunosuppression with relatively low toxicity, has gained widespread acceptance in solid organ transplantation.

In spite of this knowledge, combination therapy was only recently introduced into clinical composite tissue allotransplantation with several cases of human hand transplantation^{13,16,24,32}. In those cases tacrolimus, MMF and corticosteroids were used and found to be effective.

Corticosteroids are associated with several complications, including poor wound and bone healing², and opportunistic infections²⁵, which are particularly relevant in CTAs. Accordingly, several new drug regimens that effectively prolong CTA survival without relying on chronic corticosteroid therapy have been or are being investigated. Drugs, such as tacrolimus³, MMF⁶, rapamycin¹⁵, or FTY-720³¹ have all been tested in CTA models, either as monotherapy or in a variety of different combinations. However, the outcomes of studies that have tested monotherapies in CTA models have been disappointing. For example, rapamycin monotherapy prolonged hind-limb survival for only 9 days in a Brown Norway-to-Lewis rat model¹⁵. In contrast when rapamycin has been combined with CsA or tacrolimus, limb survival has been significantly increased¹⁵. These findings make the strong argument that the combination of calcineurin inhibitors such as CsA or tacrolimus with new drugs²⁷⁻²⁹ that target signaling pathways such as rapamycin or macrophage dependent T-cell function such as DSG, or other mechanisms such as Janus kinase inhibitors²², or FTY-720¹¹ is an effective method of providing corticosteroid-free anti-rejection therapy in CTAs.

Many studies that test the effectiveness of monotherapy immunosuppression have been performed in the rat hind-limb CTA model. In early studies, limb recipients that were treated with varying doses of azathioprine (AZA), 6-mercaptopurine (6-MP), and prednisone, died from drug-induced side effects before the onset of macroscopic signs of rejection²⁶. Although, in some cases, investigators reported long-term limb survival using CsA monotherapy^{8,9,17,19}, others have described early³⁴ and delayed^{6,20} skin rejection.

Another study reported long-term limb survival using a single large dose of tacrolimus, administered on the day of surgery (10 mg/kg) followed by weekly maintenance dosing (3 mg/kg). However, most of the animals developed *Pneumocystis carinii* pneumonia and died³. In another report, tacrolimus was given daily for 2 weeks post-transplantation at doses of 0.32-0.64 mg/kg per day i.m., and while the immunosuppressive effect observed

was similar to that seen in the CSA studies, this regimen of tacrolimus resulted in early rejection of the skin component of the limb CTA in most of the animals. Using ten times higher doses of tacrolimus administered orally, Fealy et al. found that tacrolimus significantly prolonged allograft survival and prevented rejection¹⁵. In another series of studies, MMF was shown to prevent⁶ and reverse¹⁹ established acute rejection, although in the former study animals suffered early weight loss and moderate bone marrow toxicity with long-term therapy. Finally, using the same rat hind limb CTA model, Benhaim et al.⁷ reported no significant difference between intermittent immunosuppression with CsA (25 mg/kg) or MMF (30 mg/kg), but found that tacrolimus (2 mg/kg) was significantly superior, in graft survival and lesser toxicity, to either CsA or MMF. The implication of this study was that, of the drugs studied, tacrolimus monotherapy was the only agent capable of preventing rejection of the skin component of a CTA. However, tacrolimus monotherapy did not achieve this without inevitable drug toxicity. Combined, these findings suggest that in the case of monotherapy, relatively high doses of drugs are necessary to prevent rejection across major histocompatibility barriers.

A few studies that tested the effectiveness of combination immunosuppressive therapy have also been conducted in the rat hind limb CTA model. Benhaim et al reported long-term limb survival and low toxicity in 89% of rats that received a combination of low-dose CsA (1.5 mg/kg) and low dose MMF (15 mg/kg)⁷. In the present study we found that when combined, lower doses of tacrolimus (1 mg/kg per day) and MMF (15 mg/kg per day) provided long-term limb survival and minimal toxic side effects. Throughout the duration of this 5-month study, sporadic rejection episodes were observed in these seven immunosuppressed animals. These rejection episodes correlated with sporadic episodes of self-limiting diarrhea, which could have caused erratic absorption of MMF and the resulting rejection episodes. All rejection episodes were effectively controlled by administration of tacrolimus for 7 consecutive days and returning to the bi-weekly regimen, without the dose of MMF being changed. Peripheral blood drug level measurements in two animals (in group III) confirmed that both drugs remained within therapeutic ranges and well below the blood drug levels reported in other comparable studies^{3,7}.

In rodent models, MMF is commonly reported to cause dose-dependent aplastic anemia, due to bone marrow toxicity^{1,14}, and a wasting syndrome associated with diarrhea due to gastrointestinal toxicity⁵. In our study, the only side effect that we observed was episodic diarrhea, which we attributed to the MMF. In spite of this, normal weight gain was observed in all

animals post-operatively. In rodent models tacrolimus has been reported to be nephrotoxic and hepatotoxic¹⁸. However, in this study, at the low dose of tacrolimus we used, we did not observe either of these side effects. At 30, 60, 90 and 150 days after transplantation we performed flow cytometry measurements to detect the presence of donor cells derived from the bone marrow within the CTA hind-limb and found them to be present at in our first measurement 30 day post-transplantation. These data confirm previous findings that the bone marrow within a CTA has the capacity to induce chimerism^{23,35}. We found that the mean level of chimerism (at the time of killing) in the animals that underwent rejection episodes during the length of the study was $8.6 \pm 5.8\%$, whereas the level of chimerism in the animal that experienced no rejection episodes was 2.0%. Previous studies have shown that levels as low as 1% donor cell chimerism resulting from donor stem cell engraftment can confer stable tolerance²¹. In our study, despite the presence of over 1% of donor chimerism in long-surviving animals, we found no relation between presence of donor chimerism and allograft survival. Such a finding in a rat hind limb model has been previously reported with the use of tacrolimus as monotherapy³⁷. We hypothesized that the donor cells we detected by flow typing in hosts of limb transplants could have been immunocompetent but not tolerized to the host, probably due to long term immunosuppression³⁸. In such an event, quantitative assessment of such circulating "non-tolerant" donor cells, using flow typing to reflect "engraftment" of the donor stem cells would be misleading. To determine whether flow typing reflected stem cell engraftment or a mere expansion of donor cell pool derived from the transplanted limb in the presence of immunosuppressive drugs, we examined evidence obtained during our experiments. Flow cytometry of re-suspended bone marrow from flushed femurs and tibiae of opposite (non-transplanted) limbs of hosts (at killing) revealed levels of donor chimerism similar to those found in peripheral blood. This finding suggests that the donor stem cells do engraft in hosts that are not conditioned with radiation. To enable such allogeneic donor stem cell engraftment, "geographic niches"³⁶ must first be created in the host bone marrow. This led us to hypothesize that such "niches" could have been created by either of the immunosuppressive drugs (MMF) used. We also hypothesized that sustained tolerance was not achieved despite chimerism due to dysregulation of thymic deletion or peripheral suppression mechanisms secondary to prolonged immunosuppression. We are currently investigating these mechanisms.

In conclusion, the ideal immunosuppressive strategy would be a combination of drugs that are selective and specific in function, synergistically ac-

tive for maximal effectiveness and free of toxic side effects. The present study has shown that a combination of tacrolimus and MMF provides effective long term limb and skin survival. The possibility of reducing the doses of each of these drugs afforded by administering them in combination reduced the incidence of toxic side effects. The fact we were able to achieve these results without the need for corticosteroids presents a promising therapeutic alternative for the future of clinical CTAs. In future studies, we plan to use the same low-dose combination, corticosteroid-free regimen in a large animal model (swine/primate) in an attempt to duplicate the same high level of effectiveness and low toxicity.

Based on the findings from this study, we conclude that the toxicity that is normally associated with large doses of immunosuppressive drugs and corticosteroids can be avoided. Combination immunosuppression with low dose tacrolimus and MMF prolonged CTA survival indefinitely, without the need for corticosteroids. Further studies using tacrolimus and MMF in combination with new compounds need to be conducted to further reduce immunosuppression toxicity.

Toxic side effects of immunosuppressive drugs are the reason that prevents the widespread use of composite tissue allotransplantation as a reconstructive procedure. Lowering side effects of the drugs by combining them, and at the same time prolonged survival is one of the goals in transplantation. Also, successfully eliminating the need for corticosteroids to prevent rejection will diminish important side effects such as impaired wound and bone healing and opportunistic infections.

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Chapter 4

Bone quality in rat composite tissue allo- grafts using steroid-free immunosuppression

SUBMITTED

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INTRODUCTION

Transplantation of composite tissue allografts (CTAs) from cadaveric donors offers an attractive alternative treatment option to traditional reconstructive methods for repairing large tissue defects resulting from injury, tumor extirpation and congenital defects. In spite of its promising potential CTA has not yet been widely applied in the clinical setting. This is largely due to the risks associated with the life-long immunosuppressive drugs required to prevent graft rejection in these procedures.

While most immunosuppression related complications are systemic, some will also adversely affect the transplanted graft itself. In cases of CTAs that incorporate bone (e.g. hand, femur and knee joint) these complications can include poor bone healing and bone loss leading to osteoporosis and possible spontaneous fracture. In these CTA procedures, bone strength and healing are central to the long-term functional outcomes and thus the success of these procedures¹⁻³.

In clinical organ transplantation immunosuppression related bone complications are not uncommon and are primarily caused by the corticosteroid component of the drug regimens used⁴⁻⁶. Recognizing these and other complications attempts have been made to reduce or eliminate the use of corticosteroids in transplant protocols. Recent studies showed that in selected clinical cases complete corticosteroid avoidance can be achieved in a safe and effective manner using Tacrolimus and Mycophenolate Mofetil (MMF) immunosuppression regimens^{7,8}. A recent study in a rat CTA model showed that corticosteroid-free immunosuppression could prevent rejection⁹. The purpose of the present study was to determine the effect a "steroid free" immunosuppressive regimen, using Tacrolimus and MMF, has on bone quality and healing.

MATERIALS AND METHODS

Tibiae from 20 age-matched Wistar Furth (WF) (RT1A^u) rats and ACI (RT1A^b) were used in the study (Figure 1). Tibiae were harvested from native animals and from those that underwent hind limb transplantation. Animals that received a hind limb transplant were given tacrolimus-mycophenolate mofetil (MMF) combination immunotherapy to prevent rejection. These animals were assessed daily for body weight and signs of rejection. To measure bone strength, bone acoustic velocity (v) was measured using a longitudinal ultrasound transmission technique while density (ρ) was mea-

sured using Archimedes' principle. The longitudinal elastic coefficient (E) was calculated ($E = \rho \times v^2$), as an indication of bone stiffness.

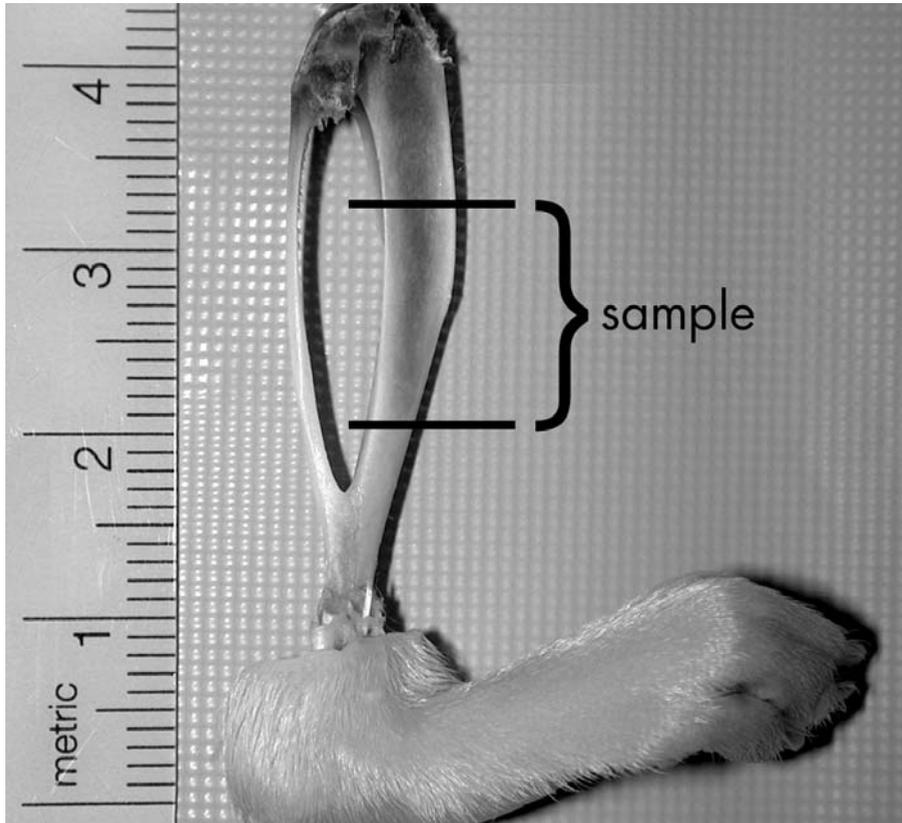


Figure 1. Segment of tibia used for bone quality measurements.

Animal care: Animals were kept in separate cages in temperature (24°C), light (12 hrs/day) and airflow regulated rooms. They were provided with a balanced rodent diet and water ad libitum. Animals were anesthetized for all surgical procedures using sodium pentobarbital (50 mg/kg, i.p.) and sterile technique was used for all survival surgeries. Upon completion of the experiments, rats were euthanized with an overdose of sodium pentobarbital. The study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86-23).

Animal model: Animals and experimental protocol. Adult male WF and ACI rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals that were used as recipients and donors for transplantation were age-matched, weighing 200-250g at the time of transplant, and had a major histocompatibility complex mismatch (WF: RT1A^u and ACI: RT1A^b).

Twenty rats (ten WF recipients and ten ACI donors) were used for hind limb transplantation and their tibiae were allocated into four groups. After transplantation animals were treated with steroid free immunosuppressive therapy (drugs and doses provided below) to prevent rejection and followed-up for 5 months. Three of the ten animals that received hind limb transplants did not complete the study period and therefore groups consisted of seven tibiae. Furthermore at the end of the study tibiae were harvested from naive WF and ACI rats. These served as control groups. These non-transplanted, non-immunosuppressed control animals were age-matched with the transplanted ACI and non-transplanted WF experimental animals, 5-months post-transplant.

Group 1: WF pre-transplant (n = 7). Tibiae harvested from the right hind limb of WF rats at the beginning of the study, at the time of transplantation.

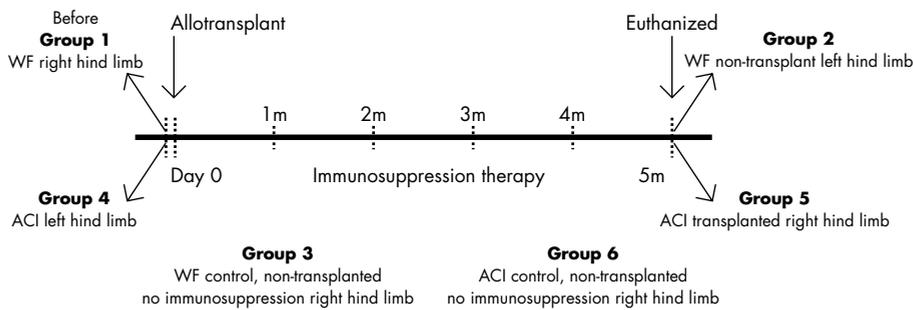
Group 2: WF post-transplant (n = 7). Tibiae removed at the end of the study from left (contralateral) hind limbs from animals that had received a right hind limb allotransplant. These limbs were not transplanted, but the tibiae had been exposed to five months of systemic immunosuppression.

Group 3: WF control (n = 7). Tibiae harvested from naïve WF rats.

Group 4: ACI pre-transplant (n = 7). Tibiae harvested from non-transplanted left hind limbs of ACI rats at the beginning of the study.

Group 5: ACI post-transplant (n = 7). Tibiae harvested from transplanted ACI hind limbs at the end of the study. These tibiae had been transplanted and exposed to five months of systemic immunosuppression.

Group 6: ACI control (n = 7). Tibiae harvested from naïve ACI rats.



Hind limb transplantation: Donor surgery. A circumferential skin incision was made just proximal to the mid-thigh area. The femoral artery, vein and nerve were dissected and the individual muscle groups of the hind limb were identified and divided as proximally as possible to their tendinous origins. Care was taken to not injure the profunda femoris vein. The sciatic nerve was identified, dissected free and divided. The donor rat was then anticoagulated with Heparin (50 U) (Elkins-Sinn, Inc. Cherry Hill, NJ), injected intravenously into the dorsal penis vein. After 10 min, the femoral artery was clamped as proximally as possible and cannulated with a 24-gauge catheter.

The limb was flushed with a solution of heparinized Ringer's Lactate (1 U of heparin per 1 ml of Ringer's solution) through the cannulated artery. The vascular flushing was maintained for 10 min until the backflow from the venous stump was clear. The femoral vein was divided, as proximally as possible. The femur was exposed and divided transversely at the mid-shaft using a handle saw. The dissected limb was then isolated and immediately placed in cold Ringers lactate in preparation for transplantation.

Recipient surgery. The operative procedure to remove the recipient limb was similar to that described for the donor except that the recipient was not anticoagulated and all the neurovascular structures were cut as distally as possible to allow for maximum length during the anastomosis of the donor limb.

The bone was fixed using a 2.5 mm Kirschner wire (1.5 cm in length) inserted intramedullary. The femoral vessels and the nerves were anastomosed using microsurgical technique (10-0 Nylon). The muscles and tendons were approximated using interrupted suture (5-0 Nylon) and the skin was closed in layers using interrupted absorbable suture (5-0 Vicryl).

Recipient rats were then returned to their cages where they were allowed to recover from anesthesia. For pain relief, ketoprofen (3-5 mg/kg; i.m.) was administered twice a day over the first three days and thereafter as needed

if animals display signs of distress. To prevent automutilation (chewing) of the insensitive, transplanted limb a solution (Butler® bitter safe mist, Columbus, Ohio) was sprayed on the transplant area three times daily for the first 8 weeks.

Immunosuppressive therapy: Animals that received a hind limb allo-transplant were immunosuppressed using a combination of Tacrolimus and Mycophenolate mofetil (MMF). Tacrolimus (Prograf 5mg/1ml ampules, Fujisawa USA, Deerfield, IL) was diluted with 5% Dextrose and administered daily in a dose of 1 mg/kg i.p. for 14 days, and twice a week 1 mg/kg i.p. thereafter until the endpoint of the study. MMF (CellCept, 500mg tablets, Roche Laboratories Inc, Nutley, NJ) was reconstituted with saline solution and given daily 15 mg/kg/day orally. During rejection episodes the dosage of tacrolimus was increased to 1 mg/kg/day for a period of one week, and subsequently returned to a dosage of 1 mg/kg biweekly.

Visual assessment of rejection: Transplanted limbs were observed daily for signs of rejection (edema, change of color and necrosis of the skin). Visual scoring criteria as previous described by Zdichavsky were used to clinically assess graft rejection¹⁰. Time of rejection was defined as the day when either, the softened surface of skin could be wiped away with a gentle touch or when the entire surface was hard and scarified with hair loss.

Histological assessment of rejection: Skin and muscle biopsies from the transplanted limbs were taken at 14 days and monthly post-transplant using a 2 mm punch. All animals were followed for 5 months or until the limb rejected. Tissues from skin and muscle were harvested, fixed in 10% buffered formalin, sectioned and stained with hematoxylin and eosin for microscopic examination. A pathologist read all the slides in a blinded fashion, and scored them using a histological grading scale established for rejection¹¹.

Bone quality measurements: Bone quality was evaluated by calculating the bone biomechanical properties with data obtained from acoustic velocity and bone density measurements of bone samples from tibiae of transplanted and non-transplanted contralateral hind limbs¹²⁻¹⁴. The acoustic velocity was measured using a longitudinal ultrasound transmission technique and bone density using Archimedes' principle¹⁵.

Bone samples previously stored in 10% buffered formalin were dissected free of soft tissue and the bone marrow was flushed out before the measurements were performed. Each of the respective tibiae bones were cut perpendicular to their long axis using a low speed diamond saw (Beuler Isomet; Lake Bluff, IL). A second cut parallel to and approximately 1 cm from the first cut was made and each specimen (Figure 1) was placed between an ultrasound transmitting and receiving transducers (Panametrics SmH2, Waltham, MA). With the cortical bone pathway to be measured centered on the transducer surfaces, a 15 V square wave signal was applied to the transmitting transducer using a function generator (Model 3011, BC Precision; Chicago, IL). This input signal was also connected to one channel of a digitizing oscilloscope (model 54501A, Hewlett Packard; San Jose, CA). The output from the receiving transducer was connected to a second channel of the oscilloscope. By comparing the onset of the input and received signals, the transmission time, Δt , of the ultrasound energy crossing through the specimen could be determined. The length of the bone segment (l) was measured using a micrometer (M.G. Tool Company, NY). The acoustic velocity (v) was then calculated as follows: $v = l/\Delta t$, where l is the length of the specimen spanning the ultrasound transducers. All the measurements were carried out at room temperature. Results of acoustic velocity measurements were expressed in m/s.

Bone density measurements were obtained according to Archimedes' principle. The mid-diaphyseal cortical bone specimens previously used in the acoustic velocity measurements were stored in distilled water in a 360 mm Hg vacuum for 30 minutes. Next, the hydrated specimen was weighed both in and out of the water bath. Bone density measurements were calculated as follows: density (ρ) = $(A/A-B) \times P$, where A is the weight of the hydrated bone, B is the weight of the hydrated bone submerged in water, and P is the density of distilled water at a given temperature. $A-B$ is the equivalent to the volume of the bone specimen. Results of bone density measurements are expressed in kg/m^3 .

In order to further assess the potential differences in bone quality of the immunosuppressed and control transplanted radial bones, a longitudinal elastic coefficient (E) was calculated for the tibial bone specimen using the relationship: $E = \rho \times v^2$. Where ρ is the tibial bone specimen density and v the measured acoustic velocity¹⁴. Assuming that the acoustic wave pathway in bone is homogeneous, the elastic coefficient represents the intrinsic longitudinal stiffness or compressive strength of the specimens. Although no clear quantitative relationship exists between the elastic coefficient or mod-

ulus of bone and its absolute mechanical strength¹⁶⁻¹⁸, recent studies show a relatively strong correlation between its modulus and ultimate strength¹⁹.

Statistics: Values are expressed as the mean values \pm SD. Changes in density, ultrasound velocity and elasticity of samples were assessed for statistical significance using an Analysis of Variance (ANOVA) followed by post-hoc paired t-test when comparing pre- and post-transplant groups and a post-hoc unpaired t-test when comparing post-transplant groups with the control groups. Differences were considered significant when the p value was less than 0.05.

RESULTS

Animals: Seven of ten rats completed the study and were sacrificed at five months. Of these seven animals one experienced no rejection episodes, four had a single rejection episode, and two rats had multiple rejection episodes. All rejection episodes were successfully reversed by adjusting the dose of immunosuppressive drugs for a period of one week. Three of the ten animals that received hind limb transplants did not complete the study period due to unknown causes in one and auto mutilation of the transplanted limbs in the other two. The transplanted limb of the third animal experienced an episode of acute rejection and the limbs of the other two rats did not.

Histopathology: The transplanted hind limbs of the three animals that did not complete the follow up period showed no histological signs of rejection at the time of death. However, in three of the seven animals that completed the study, mild rejection of the transplanted limbs was found as illustrated by mononuclear dermal infiltration in skin biopsy samples. In the rest of the four remaining animals histology was normal without signs of rejection.

Bone quality measurements: Bone quality was assessed using bone density and acoustic velocity measurements and by calculating the elastic coefficient. The results of these measurements are summarized in Figures 2, 3 and 4.

Density (ρ): Figure 2: In both the WF and ACI groups the mean bone densities pre-transplant were not statistically different from the mean bone

density post-transplant (Group 1 vs Group 2: $2015,8 \pm 37,4$ vs $2064,7 \pm 56,2$ kg/m³ and Group 4 vs Group 5: $1969,3 \pm 79,6$ vs $2041,4 \pm 67,8$ kg/m³). There was also no significant difference between the mean bone density post-transplant and the mean density of the age-matched control groups of both WF and ACI tibiae (Group 2 vs Group 3: $2064,7 \pm 56,2$ vs $2101,6 \pm 17,9$ kg/m³ and Group 5 vs Group 6: $2041,4 \pm 67,8$ vs $2089,0 \pm 5,3$ kg/m³).

Acoustic velocity (v): Figure 3: The difference between the mean acoustic velocity of the WF tibiae pre-transplant ($3761,2 \pm 189,4$ m/s) and post-transplant ($3849,8 \pm 161,6$ m/s) was not significant. The difference between the mean acoustic velocity of the WF tibiae post-transplant ($3849,83 \pm 161,6$ m/s) and the WF control group ($4165,5 \pm 61,1$ m/s) was significant $p < 0,001$. The difference between the mean acoustic velocity of the ACI tibiae pre-transplant ($3634,3 \pm 137,0$ m/s) and post-transplant ($3799,8 \pm 99,1$ m/s) was significant $p < 0,05$. The difference between the mean acoustic velocity of the ACI tibiae post-transplant ($3799,8 \pm 99,1$ m/s) and the ACI control group ($4110,5 \pm 102,7$ m/s) was significant $p < 0,0001$.

Elastic coefficient (E): Figure 4: The mean elastic coefficient of the non-transplanted WF tibiae pre-transplant, Group 1, ($28,6 \pm 3,2$ GPa) was not significantly different from post-transplant, Group 2, ($30,6 \pm 2,4$ GPa). However, when the mean elastic coefficient of the WF tibiae post-transplant, Group 2, was compared with the WF control, Group 3, there was a very significant difference (Group 2 vs Group 3: $30,6 \pm 2,4$ vs $36,5 \pm 1,0$ GPa; $p < 0,0001$).

The mean elastic coefficient of the transplanted ACI tibiae pre-transplant, Group 4, ($26,1 \pm 2,5$ GPa) was significantly ($p < 0,05$) different from post-transplant, Group 5, ($29,5 \pm 1,7$ GPa). However, when the mean elastic coefficient of the ACI tibiae post-transplant, Group 5, was compared with the ACI control, Group 6, there was a significant difference (Group 5 vs Group 6: $29,5 \pm 1,7$ vs $35,3 \pm 1,7$ GPa; $p < 0,0001$).

Bone healing: At the time of autopsy all transplanted limbs showed union of the femoral bones as determined by visual inspection.

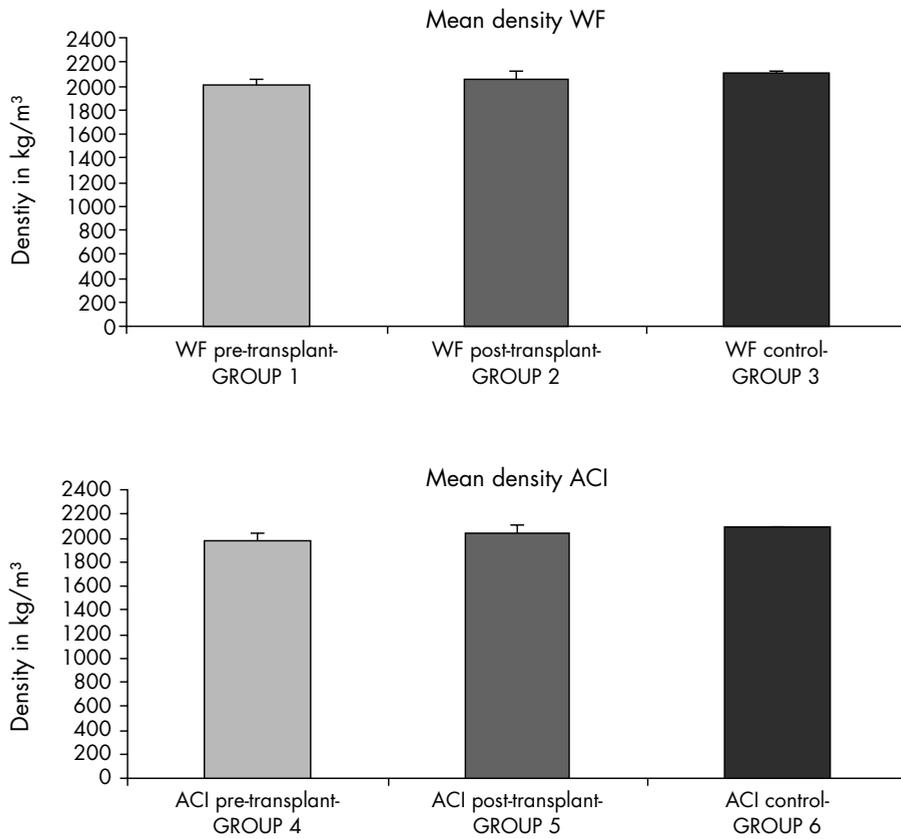


Figure 2. Graph showing the density results (mean \pm SD) obtained from WF and ACI bone samples pre- and post-transplant and controls. There was no significant difference between groups.

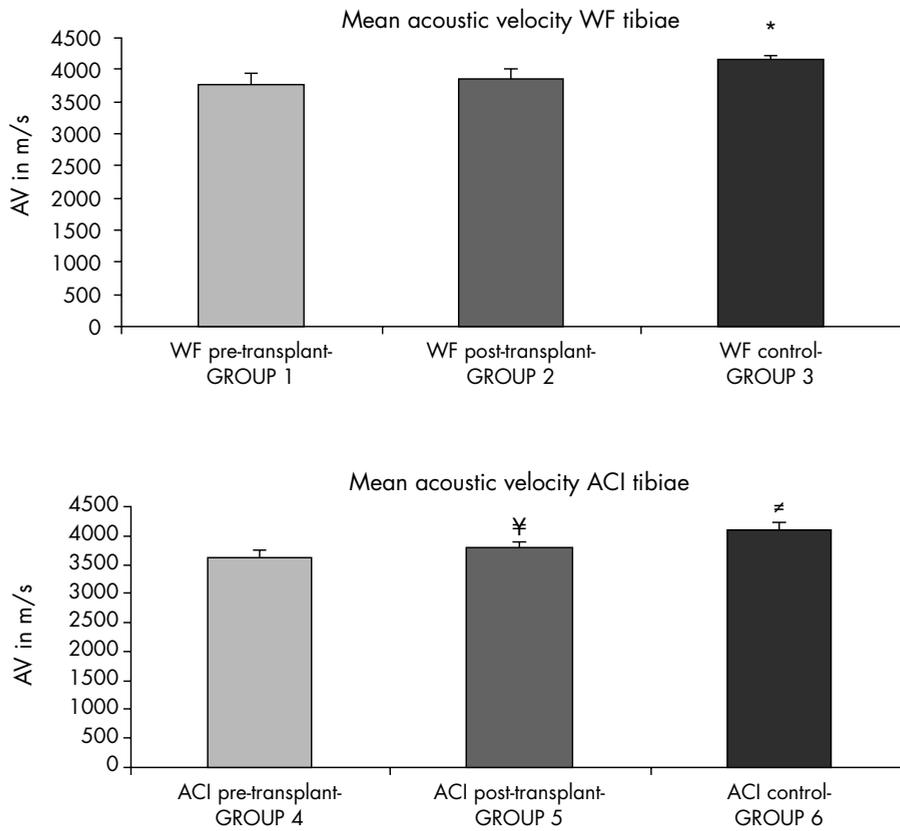


Figure 3. Graph showing the acoustic velocity results (mean \pm SD) obtained from WF and ACI bone samples pre- and post-transplant and controls.

*: WF post-transplant vs. WF control $p < 0,001$

¥: ACI pre-transplant vs. ACI post-transplant $p < 0,05$

≠: ACI post-transplant vs. ACI control $P < 0,0001$

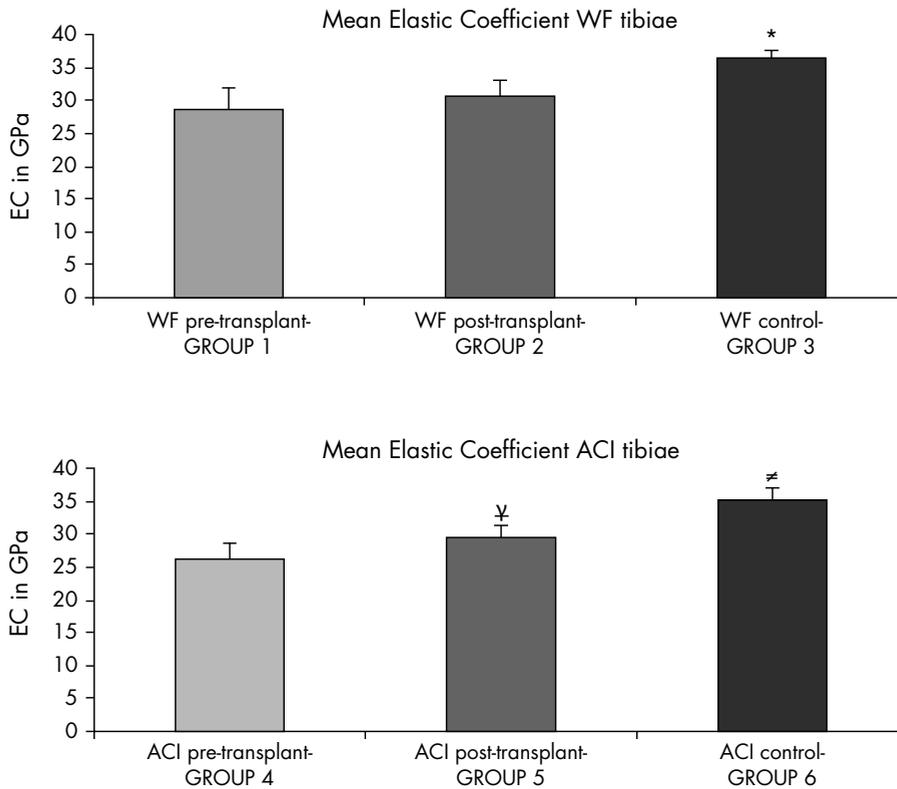


Figure 4. Graph showing the elastic coefficient results (mean \pm SD) obtained from WF and ACI bone samples pre- and post-transplant and controls.

*: WF post-transplant vs. WF control $p < 0,0001$

†: ACI pre-transplant vs. ACI post-transplant $p < 0,05$

‡: ACI post-transplant vs. ACI control $p < 0,0001$

DISCUSSION

There has been increasing interest in the transplant community regarding the reduction and avoidance of corticosteroids. Among the well-established side effects of corticosteroids are post-transplantation bone loss and increased fracture risk²⁰⁻²². Recent advances with newer immunosuppressive agents and combination therapy, particularly when combining tacrolimus and MMF, have successfully allowed for more rapid discontinuation or avoidance of corticosteroids in organ transplant recipients²³⁻²⁶.

In this study we investigated the effect “corticosteroid-free” immunosuppression, using effective doses of tacrolimus and MMF, had on bone quality and healing in a rat hind limb CTA model. We found that a combination of low dose tacrolimus (1 mg/kg/day) and MMF (15 mg/kg/day) allowed for the elimination of corticosteroids while preventing hind limb CTA rejection. Throughout the duration of this 5-month study, sporadic rejection episodes were observed in the immunosuppressed animals. These rejection episodes correlated with sporadic episodes of self-limiting diarrhea, which could have caused erratic absorption of MMF and the resulting rejection episodes. All rejection episodes were effectively reversed by adjusting the dose of tacrolimus for a period of seven consecutive days and then returning to the bi-weekly regimen, without changing the dose of MMF.

In rodent models, MMF is reported to cause hypoplastic anemia due to bone marrow depression²⁷, and a wasting syndrome associated with diarrhea due to gastrointestinal toxicity²⁸. In this study episodes of diarrhea were observed, which were ascribed to MMF. Despite these episodes of diarrhea, all animals gained weight post-transplant.

The principal adverse effect associated with tacrolimus treatment is reported to be nephrotoxicity²⁹. In rat models tacrolimus in a dose of 3 mg/kg/day is reported to impair renal function due to medullary injury³⁰⁻³². However in this study, at the low dose (1 mg/kg/day) used, this side effect was not observed.

In the experiments described here we investigated, for the first time, bone quality in a rat hind limb CTA model while using a steroid free immunosuppression regimen. We demonstrated that the acoustic velocity and the elastic coefficient of transplanted allogeneic and non-transplanted autologous bone was significantly lower when compared with age-matched non-transplanted, non-immunosuppressed control animals. Since this effect occurs in both the allogeneic transplanted and the autologous non-transplanted bone, it is most likely caused by the immunosuppressive regimen used in our study.

The effects immunosuppressive therapy has on bone density and quality have been well documented in the field of organ transplantation³³. From these studies it is known that immunosuppressive therapy is one of the leading contributing factors to post-transplantation osteoporosis. Most studies have looked at corticosteroids as the leading cause of this reduction in bone quality post-transplant, and only very few studies investigated tacrolimus and MMF in this respect. In a rat model tacrolimus was reported³⁴⁻³⁶ to cause an accelerated bone remodelling with resorption far in excess of formation, leading to a loss of bone volume. Another study showed that

MMF (30 mg/kg/day) maintained bone volume, but decreased in-vivo osteoblastic bone mineral metabolism in rats³⁷.

A recent report describing long-term follow-up of the first clinical vascularized knee allotransplants showed that 50 percent of the cases suffered graft dysfunction due to late rejection³⁸. In the present study, since rejection episodes were observed in some of the immunosuppressed animals, we wondered if these might have adversely affected bone quality and thus our results. Interestingly in the one rat that had no rejection episodes we measured the lowest bone density and elastic coefficient in the tibia of the transplanted ACI hind limb when compared with the rest of the tibiae in its own group (Group 5: ACI post-transplant). Furthermore in the rat that suffered the most episodes of rejection bone density, acoustic velocity and elastic coefficient of the transplanted tibia were all higher than the mean result in this group (5). These observations do not support the hypothesis that rejection adversely affects bone quality. The small number of animals in this group is of course a limitation and warrants further studies focusing on the effect of rejection on the quality of allotransplanted bone.

In the experiments described here we found that tacrolimus and MMF had no adverse effects on bone healing in our rat model. This correlates with the findings of Voggenreiter et al. in which in a similar rat model they found that tacrolimus (1 mg/kg bodyweight) did not effect fracture healing³⁹. They observed that after 4 weeks of systemic tacrolimus administration in a rat tibial fracture model bone healing, as determined by biomechanical and histological measurements, was normal. When evaluating these findings one must take into consideration the relatively short duration of the study. There are no reports on MMF and fracture healing.

In conclusion, we found that elimination corticosteroids from the tacrolimus/MMF immunosuppression regimen used in this study effectively prevented rejection, did not alter bone healing however, did negatively impact bone quality. Based on these findings, when performing clinical CTA procedures that include bone, it is important to closely monitor bone quality. Future studies are needed to investigate other immunosuppression regimens that effectively prevent CTA rejection while at the same time do not compromise bone quality.

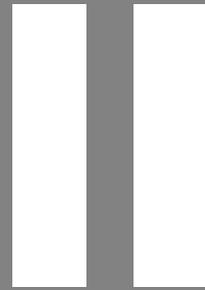
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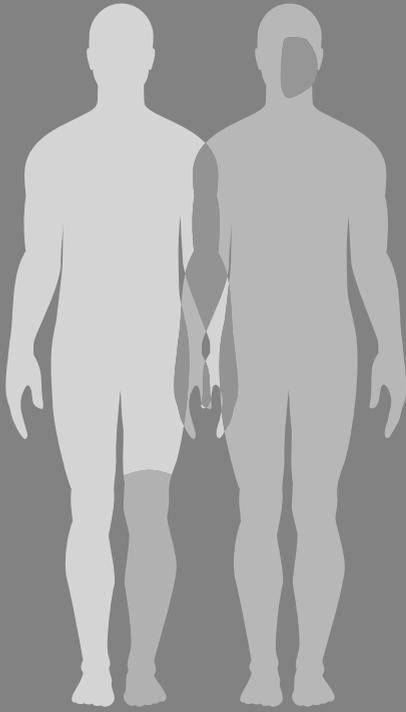
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Part





Chapter 5

Mixed allogeneic chimerism: past, present and prospects for the future

TRANSPLANTATION 2001; 72(8): S32-S42

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S.T. Ildstad

INTRODUCTION

Advances in microsurgical techniques, antibiotics and effective immunosuppressive agents have made solid organ transplantation a routine clinical procedure for the treatment of end-stage organ failure. However, with these advances new challenges have emerged. The life-long use of immunosuppressive drugs is associated with an increased occurrence of neoplasms, opportunistic infections, and end-organ toxicity including renal failure^{1,2}. Despite improved immunosuppressive regimes, effective prevention of chronic graft rejection is not achieved and long-term survival of all grafts is limited³. Chronic rejection is the major cause of late allograft loss^{4,5}. Even with the use of modern immunosuppression, approximately 35% of heart, liver and cadaveric renal allografts are lost within five years⁶. Five-year survival of lung grafts is 42% and 35% for pancreas grafts⁶.

The induction of transplantation tolerance is one of the major goals in transplantation immunology. One of the most effective and best-studied approaches to achieve this goal is through bone marrow transplantation (BMT), which results in hematopoietic stem cell (HSC) chimerism. However, the induction of chimerism by BMT has its own risks, which must be reduced or eliminated for this procedure to become a clinical reality. This review will discuss the use of BMT for induction of chimerism and donor-specific tolerance with special emphasis on approaches to overcome the current limitations.

HISTORY

In 1945, Owen first described a naturally occurring state of hematopoietic chimerism when he demonstrated the presence of red blood cell chimerism in dizygotic Freemartin cattle twins that share a common placental circulation⁷. In humans, red blood cell chimerism was similarly reported in a dizygotic twin several years later⁸. Shortly thereafter, Anderson and Billingham observed that reciprocal skin grafts between Freemartin chimeras were accepted, while they were rejected by other cattle⁹. Following these observations, Billingham, Brent, and Medawar performed a classical series of experiments and for the first time actively induced hematopoietic chimerism by inoculation of a tissue suspension containing hematopoietic cells into fetal mice¹⁰. These mice demonstrated acquired donor-specific tolerance to subsequent skin grafts after birth. In 1955, Main and Prehn achieved donor-specific tolerance for the first time in adult animals by injecting bone marrow into mice conditioned with irradiation¹¹. Since then, donor-specific

tolerance induced by hematopoietic chimerism has been accomplished in numerous experimental models including rodents, large animals and primates¹²⁻¹⁶.

In humans, the first successful bone marrow transplants were performed over 40 years ago between genetically identical twins¹⁷. Transplantation of bone marrow between major histocompatibility complex (MHC)-disparate allogeneic recipients posed more of a barrier in that graft-versus-host disease (GVHD) was a formidable challenge. This led to the development of the human leucocyte antigen (HLA)-typing, making identification of MHC-compatible related and unrelated donors possible¹⁸. The importance of genetic histocompatibility antigens in transplantation was highlighted by the fact that the occurrence and severity of GVHD was directly correlated with the degree of mismatch between donor and recipient^{19,20}. In humans, the first association between chimerism and tolerance was reported in 1985 by Knobler et al. for skin allografts²¹. He reported a patient treated for severe aplastic anemia with allogeneic BMT that developed severe chronic mucocutaneous GVHD which resulted in persistent non-healing ulcers. Allografted split-thickness skin grafts from the same donor as the bone marrow were taken to cover these ulcerations. The donor-specific allografted skin transplants were accepted and unresponsiveness to donor-type alloantigens was demonstrated in vitro. Later, Sayegh et al. reported the drug-free acceptance of renal allografts in patients who received BMT to treat hematopoietic malignancies and later developed renal failure²². A subsequent kidney transplant from the same donor as the bone marrow maintained function without requiring significant immunosuppression for anti-rejection therapy.

At present, BMT is not routinely used for the induction of tolerance to solid organs, cellular grafts or composite tissues grafts, due to the severe side effects and possible complications associated with conventional BMT. GVHD, failure of engraftment, and the toxicity of conditioning required for engraftment of donor bone marrow have prevented the widespread clinical application of chimerism to nonmalignant disease. Methods to make BMT safer will allow the more widespread application of chimerism to induce tolerance and treat nonmalignant disease states such as autoimmune disease.

MIXED ALLOGENEIC CHIMERISM

Deletional tolerance is induced by HSC chimerism: When bone marrow is transplanted in a conditioned recipient the pluripotent HSC en-

grafts in the recipient and produces multiple lineages, a state referred to as multilineage macrochimerism²³. A new immune system is established in the recipient. Two types of chimeras exist: fully allogeneic in which the donor fully replaces the recipient hematopoietic system, and mixed allogeneic chimeras in which the donor and recipient HSC coexist. Hematopoietic cell progeny from both the recipient and the donor migrate to the thymus where they mediate the deletion of both recipient- and donor-reactive T cells via negative selection. Two critical lineages contribute to this event: pre-T cells and bone marrow derived dendritic cells (Fig. 1A)²⁴. Consequently, deletion results in a robust form of tolerance since all donor-reactive T cells are eliminated and thus the recipient recognizes donor grafts as self. The coexistence of genetically distinct donor and recipient tissues/cells in one animal or individual is referred to as chimerism and is one of the most effective and best-studied ways to establish donor-specific tolerance.

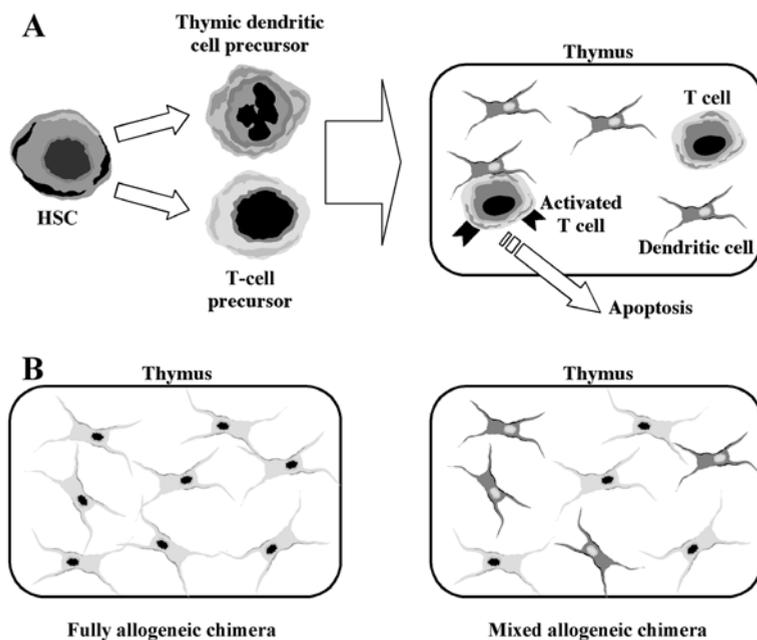


Figure 1. (A) HSC derived T-cell precursors and thymic dendritic cell precursors migrate from the bone marrow to the thymus. T cells undergo thymic selection upon interaction with thymic dendritic cells. Those that respond too strongly to antigen in the thymus are deleted by a process termed apoptosis, or programmed cell death. (B) In the thymus of fully allogeneic chimeras only donor-derived dendritic cells are present, whereas in mixed allogeneic chimeras both recipient and donor dendritic cells are present.



Figure 2. The mythological chimera is a creature consisting of tissues from several different species. The statue is a copy of the original Etruscan bronze sculpture created in the 5th - 4th century BC, which is located in the Museo Archeologico Nazionale, Florence, Italy. Photography is taken in Arezzo, Italy, by M. Brouha.

In Greek mythology the chimera is a creature consisting of tissues from several different species (Fig. 2). In the context of transplantation two different types of chimerism must be differentiated: microchimerism and macrochimerism. Microchimerism arises naturally through transplantation of solid organs or cellular grafts due to migration of passenger leucocytes present within the graft²⁵. It does not require conditioning of the recipient and pluripotent HSC do not routinely engraft. In microchimerism levels of donor-specific cells are usually very low and typically only consist of class II⁺ dendritic cells detectable by molecular techniques or rare event cell sorting²⁵. It is debated whether microchimerism is responsible for tolerance or a side effect of organ allograft acceptance²⁵. Allograft rejection with microchimerism and long-term allograft survival without microchimerism have been reported, suggesting that microchimerism is not required for induction and maintenance of tolerance but rather associated with graft survival²⁶⁻²⁸. Macrochimerism occurs when bone marrow is transplanted and engraftment of the pluripotent HSC occurs, giving rise to the production of all its lineages²³. The level of macrochimerism and the degree of tolerance do not seem to be correlated, because levels of chimerism as low as 1% are

sufficient to induce robust donor-specific tolerance to skin and solid organ allografts^{12,29,30}. The tolerance associated with HSC macrochimerism is so robust that it can even be achieved across closely related species (i.e. rat / mouse)^{29,31,32}. Four different types of macrochimerism exist. Syngeneic chimerism refers to the situation in which donor bone marrow is transplanted from a genetically identical recipient. Allogeneic chimerism refers to an individual transplanted with bone marrow from a genetically different donor within the same species. In fully allogeneic chimeras virtually all bone marrow cells are donor-derived, whereas in mixed allogeneic chimeras bone marrow cells of recipient and donor coexist. Mixed allogeneic chimerism can be established if the recipient is incompletely myeloablated before BMT or when a fully myeloablated recipient is reconstituted with a mixture of syngeneic and allogeneic bone marrow (Fig. 3)¹². In addition, xenogeneic chimeras are prepared by bone marrow cells from a different species donor³². Chimeras prepared by BMT and conditioning show tolerance to donor-specific allografts, including skin, heart, lung, and pancreatic islets, while retaining the immunocompetence to reject an MHC-disparate third-party donor^{12,31,33,34}.

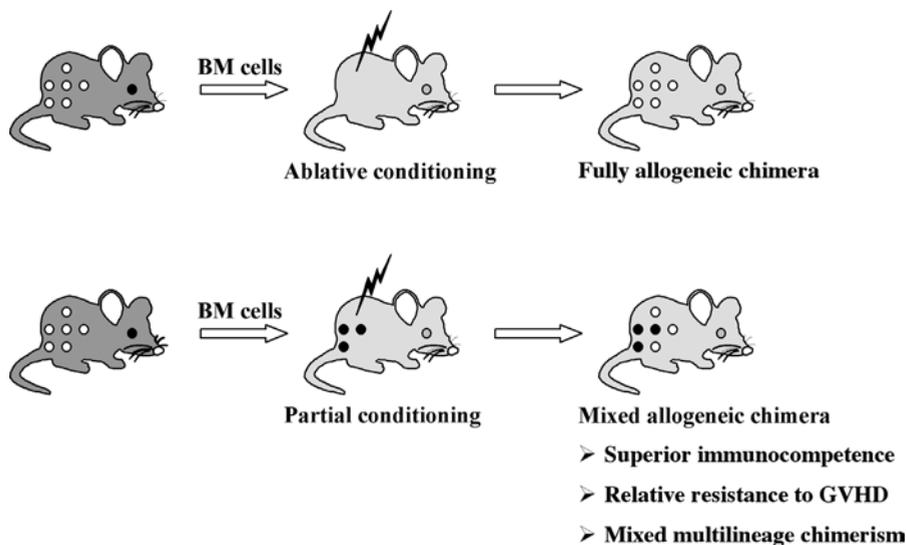


Figure 3. In fully allogeneic chimeras, the recipient hematopoietic compartment is replaced by donor-derived HSC. In mixed allogeneic chimeras, both recipient and donor HSC coexist.

Advantages of mixed chimerism: Mixed allogeneic chimeras have a number of advantages over fully allogeneic chimeras: (1) Mixed allogeneic chimeras exhibit superior immunocompetence for primary immune responses because of the presence of recipient-derived antigen presenting cells (APC)^{35,36}. This can be explained by the requirement of mature T cells to interact with “self” APC to be able to recognize antigen and initiate a primary immune response. T-cell precursors emerging from the bone marrow migrate and mature in the allogeneic recipient thymic environment. The T cells are educated to recognize non-bone-marrow derived thymic stromal MHC as self³⁷⁻⁴¹. Mixed chimeras produce both donor- and recipient-derived APC, the latter of which is able to interact with mature T cells from either origin that underwent thymic maturation. In full chimeras all APC are donor derived (Fig. 1B). However, the newly developing T cells are positively selected to recognize and interact with APC expressing recipient MHC class I antigens, which are not present on the donor-derived APC (Figs. 1A and 1B)⁴². As a result, fully allogeneic chimeras are relatively immunoincompetent. Mixed chimeras exhibit superior ability to eliminate viral infections and produce antibody compared to fully allogeneic chimeras. In fact, rat T lymphocytes that develop in mouse chimeras are tolerant to the recipient mouse strain and also exhibit similar preferential restriction to recipient APC. As a result, primary immune responses in mixed xenogeneic chimeras are also superior⁴³. (2) Mixed chimeras are also relatively resistant to GVHD compared with fully allogeneic chimeras^{30,44}. (3) Mixed chimerism can be induced with partial conditioning, thus avoiding the approximate 10% mortality associated with fully ablative conditioning. These three advantages may make mixed chimerism the preferential approach to induce deletional tolerance to solid organ allografts and for the use of BMT in treatment of nonmalignant diseases such as autoimmunity and hemoglobinopathies.

Conditioning regimens: Engraftment of bone marrow usually requires some form of recipient conditioning⁴⁵. For years it was believed that fully ablative conditioning was a requirement for durable bone marrow engraftment to occur. Because there is an approximate 10% mortality from full ablation, strategies to apply chimerism to induce tolerance were not broadly attempted clinically.

Engraftment of MHC-identical bone marrow without irradiation was first achieved in mice conditioned with anti-lymphocyte serum⁴⁶. Pre-treatment of recipients with monoclonal antibodies (mAbs) against MHC class I and II antigens in mice showed similar results⁴⁷. Engraftment of fully MHC mis-

matched bone marrow grafts has now been achieved in a variety of animal models with partial conditioning using non-specific immunosuppressive agents or lymphocyte-specific interventions. Low-dose total body irradiation (TBI) and/or thymic irradiation together with immunosuppressive agents (cyclophosphamide, tacrolimus) have been successfully used to establish chimerism^{48,49}. Furthermore, the addition of mAbs (anti-CD4, anti-CD8, and anti-natural killer) to conditioning have allowed a significant reduction in the dose of irradiation⁵⁰⁻⁵². By increasing the dose of marrow, the minimum dose of TBI to obtain engraftment can be further reduced^{53,54}. By optimizing the composition of the donor marrow and targeting those factors in the recipient microenvironment that resist engraftment of allogeneic marrow, one can significantly reduce the risk of conditioning.

One potentially promising approach to establish chimerism with partial conditioning is through the conditioning of the recipient with mAbs directed to co-stimulatory molecules on T cells. T-cell activation requires antigen recognition by the T-cell receptor plus stabilization of T cell:APC interactions by co-stimulatory molecules. When very high numbers of bone marrow cells were administered to MHC-disparate mice pre-treated with α CD40L plus cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4Ig), engraftment occurred in a significant fraction of mice without any TBI⁵⁵. Although the number of bone marrow cells required for engraftment in this model could probably not be obtained from human donors, this observation highlights the importance of targeting specific effector cell populations for conditioning.

Chimerism reverses sensitization to alloantigens: The sensitized state is a major obstacle to finding donors for renal and cardiac allograft recipients⁵⁶⁻⁵⁸. To determine whether HSC chimerism would reverse sensitization to MHC alloantigens, mice were sensitized with skin grafts and sensitization was documented⁵⁶. Mice were then conditioned and transplanted with donor-specific bone marrow cells. Although a significantly higher cell dose was required to establish chimerism compared with naïve recipients, when chimerism resulted, the antidonor antibody and cellular responses were eliminated. In conditioning studies, approaches to target the recipient effector cells for alloreactivity should allow chimerism to be established with only partial conditioning, an approach that may significantly improve outcomes in sensitized recipients of organ allografts.

Graft-Versus-Host Disease: GVHD is a major complication of BMT^{45,59}. In GVHD donor T cells, B cells, and natural killer cells contained

within the allograft recognize recipient antigens as non-self and mount an immune response against the recipient's body. GVHD affects many organs, including skin, gastrointestinal tract, and liver. The incidence and severity is highly correlated with the degree of MHC mismatch between donor and recipient and the number of donor T cells transplanted^{19,20}. In humans, HLA-matched related siblings have a 30-50% chance of developing GVHD^{20,60,61}. This risk increases further to about 70% when one of the six HLA loci are mismatched^{20,61-63}. Virtually all patients develop GVHD when a mismatch in two or more loci is present²⁰. Once it was recognized that T cells were the most important effector cell subset in GVHD, laboratory and clinical protocols were developed that depleted T cells from bone marrow grafts^{19,45}. The incidence and severity of GVHD is effectively reduced in animal models and in humans when the marrow is depleted of T cells. However, transplantation of T-cell depleted bone marrow was associated with a relatively high incidence of engraftment failure and required more myeloablative conditioning for the bone marrow to engraft as compared to unmodified bone marrow^{19,45,64}. This dichotomy of engraftment and GVHD led to the following hypotheses: (1) either T cells are essential for engraftment and GVHD cannot be avoided, or (2) the cells responsible for engraftment are different from T cells, but share some markers present on T cells and are removed by the T-cell depletion procedure.

Studies showing that higher doses of highly purified HSC achieve engraftment across MHC barriers suggest that engraftment is increased by the presence of cofactors or accessory cells⁶⁵⁻⁶⁷. It has been suggested that some growth factors or interactions of HSC and T cells may play a role in stem cell engraftment⁶⁸. Furthermore, veto mechanisms may also play a role in prevention of rejection and facilitation of engraftment^{69,70}.

Other investigators evaluated which accessory cells are essential for engraftment of purified HSC in allogeneic recipients. El-Badri and Good co-administered purified HSC with T-cell depleted syngeneic marrow to determine whether the purified HSC required time to become metabolically active⁶⁶. If the HSC and recipient were MHC congenic, irrespective of minor antigen matching, the HSC engrafted readily. When the HSC was MHC disparate to the recipient, allogeneic chimerism was not established. However, the addition of a small number of allogeneic bone marrow cells to the HSC allowed engraftment of allogeneic marrow. They hypothesized that an accessory cell in marrow was required for HSC to engraft in MHC-disparate recipients. Studies from our laboratory confirmed these data. Although 1,000 syngeneic purified stem cells readily engraft in a lethally irradiated recipient, with survival of the animal, up to 10,000 allogeneic

purified stem cells fail to engraft in allogeneic recipients (Fig. 4). If the donor and recipient are matched at the MHC but disparate for minor antigens, engraftment also occurs readily (Fig. 4). These data suggested that an additional cell in marrow facilitates engraftment of physiologic numbers of HCS in allogeneic recipients.

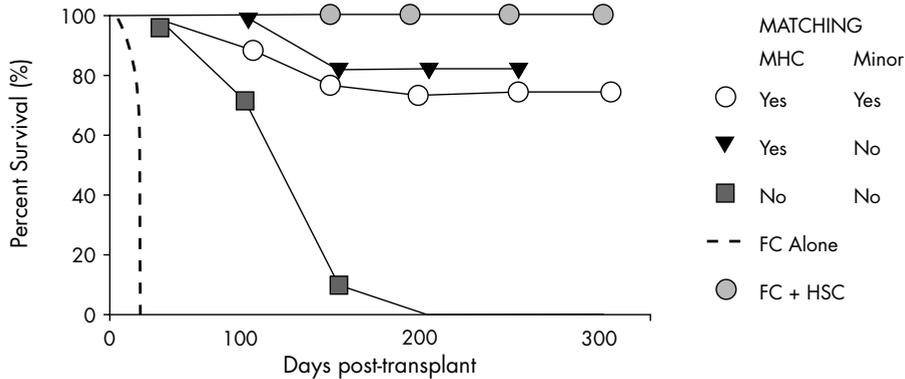


Figure 4. Purified HSC engraftment occurs readily when MHC-congenetic T-cell depleted bone marrow is co-administered (○-○). A minor antigen disparity does not influence engraftment of purified HSC (▼-▼). When HSC is MHC disparate to the recipient, durable allogeneic chimerism is not established (■-■). Animals transplanted with facilitating cells only do not radioprotect (---). The addition of facilitating cells, matched to HSC, restores engraftment of allogeneic recipients (●-●).

Kaufman et al. used a mouse model to phenotypically and functionally characterize a non-T-cell population that facilitates engraftment of highly purified stem cells across MHC barriers⁶⁵. This facilitating cell population is CD8⁺, CD3_ε⁺ and CD45⁺, class II^{dim/intermediate}, but αβ TCR⁻ and γδ TCR⁻, and comprises 0.4% of the total bone marrow. Although the majority of the CD8⁺/TCR⁻ cells in the bone marrow are CD3_ε⁻⁷¹, the biologic activity of facilitating cell is in the CD3_ε⁺ population. The addition of as few as 30,000 CD8⁺/TCR⁻ facilitating cells to 10,000 allogeneic purified stem cells restores engraftment in the allogeneic recipient and survival of the animal^{65,71}. Most importantly, the facilitating cell does not cause GVHD. Facilitating cells must be genetically matched to the donor of the bone marrow graft in order to facilitate engraftment and they do not radioprotect when transplanted without stem cells. Although once controversial, this finding has been independently confirmed and the mechanisms of action fur-

ther defined^{33,71-73}. The term facilitating cell has been incorporated into the stem cell biology vernacular and recent editorials have commented on its potential for impact clinically^{74,75}.

Facilitating cells are also required for engraftment of murine fetal liver stem cells in MHC-disparate recipients⁷⁶. Moreover, there is evidence that facilitating cells also may optimize engraftment of purified HSC transplanted into nonablated syngeneic recipients⁷³. These significant discoveries may allow one to engineer bone marrow to contain only the desired cells for rapid engraftment and avoid GVHD. Such strategies could make the use of BMT for tolerance induction a clinical reality.

Bone marrow transplantation for nonmalignant hematologic diseases, autoimmunity and immunodeficiency: Strategies to make BMT more safe by minimizing conditioning and avoiding GVHD and graft rejection would not only make BMT available for inducing allograft-specific tolerance, but could also provide treatment for patients with nonmalignant hematological diseases, autoimmunity, and immunodeficiency (Fig. 5)⁷⁷⁻⁷⁹. In these cases BMT might function as a “natural” form of gene therapy to produce a missing enzyme or protein. Various successful attempts have been made to treat nonmalignant diseases in select cases, including sickle cell anemia and thalassemia⁸⁰⁻⁸².

Evidence is emerging that numerous autoimmune diseases can be cured with allogeneic HSC transplantation. In animal models for spontaneous autoimmune disease, BMT has been shown to reverse the active immune process and in some cases even reverse pathologic changes including type I diabetes, lupus-like syndrome, glomerulonephritis, and rheumatoid arthritis^{53,78,83,84}. These observations in mice prompted clinicians to evaluate whether a clinical correlate could be identified in humans. In humans, a number of patients with rheumatoid arthritis showed remission after BMT for leukemia⁸⁵⁻⁸⁷. Other “incidental” cures by allogeneic BMT have been reported for Crohn’s colitis, psoriasis vulgaris, systemic lupus erythematosus, multiple sclerosis, and several other autoimmune diseases⁸⁸⁻⁹².

CONCLUSIONS

The association between chimerism and donor-specific tolerance has been recognized for over five decades. Despite the real problems with conventional immunosuppressive agents, induction of chimerism by BMT is not yet a clinical reality. However, new nonablative conditioning approaches and

the recent discovery of the facilitating cell may allow the use of BMT to achieve donor-specific tolerance. This could potentially revolutionize the field of transplantation and allow solid organ, cell, composite tissue and perhaps even xenogeneic grafts to be performed without the need for life-long immunosuppression. Moreover, BMT could also become a viable treatment for various nonmalignant hematological diseases, autoimmunity, and immunodeficiency.

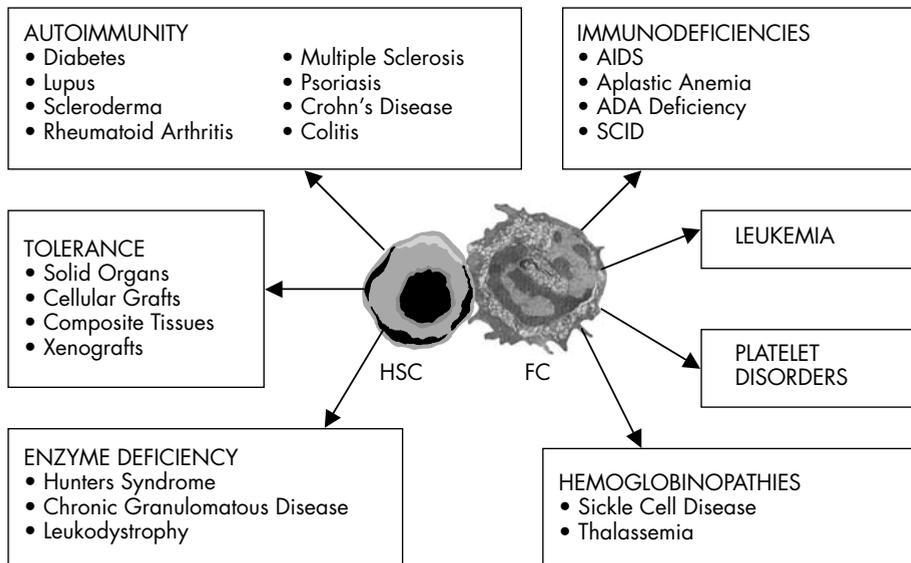


Figure 5. Potential applications of mixed allogeneic chimerism. Engineering of bone marrow with elimination of cells inducing GVHD and addition of cells promoting engraftment (facilitating cells, FC) in combination with reduced recipient conditioning could make BMT available for the treatment of many nonmalignant diseases and for the induction of transplantation tolerance.

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Chapter 6

Composite tissue allotransplantation in chimeric hosts part I. Prevention of graft- versus-host disease

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INTRODUCTION

Approximately 7 million individuals (>1 million amputees) require complex reconstructive procedures in the United States each year^{1,2}. The recent success of clinical composite tissue allotransplantation³ attests to the fact that composite tissue allografts (CTA) have tremendous potential in these life-enhancing reconstructions. However, further advancement of CTA in the clinical arena continues to be curtailed by concerns of prolonged immunosuppression⁴ to which hosts must be subjected. Efficacious, safe, and ethical clinical tolerance protocols could improve patient acceptance of CTA by providing an alternative to chronic immunosuppression. A number of methods for inducing tolerance have been described⁵, but none have been as time-tested as hematopoietic stem cell (HSC) chimerism, which is one of the oldest and best-studied approaches for establishing tolerance⁶.

Mixed allogeneic chimerism (MAC) has been shown to induce tolerance to allografts of skin⁷, heart⁸, lung⁹, pancreatic islet¹⁰, kidney¹¹, trachea¹², and composite tissue¹³ in animal and human recipients. MAC has also been shown to prevent chronic rejection in transplanted grafts, thereby overcoming another major limitation of solid-organ and cellular transplantation¹⁴. Thus, in case of CTA, the tolerance produced by MAC may be superior to graft prolongation achieved with nonspecific immunosuppressive drugs.

To date, only one study (using the rat hind-limb model) by Foster et al. has examined the potential of bone marrow (BM) and lymph node-containing CTA in causing lethal graft-versus-host disease (GVHD) in chimeric hosts that were previously made tolerant to donor antigens¹⁵. Foster et al. reported that transplantation of unmanipulated donor (ACI) limbs to [ACI→WF] chimeric hosts led to acute GVHD in one of nine (11%) animals and that irradiation of ACI limbs before transplantation prevented GVHD. The authors report here a rat hind-limb CTA model for acute GVHD, in which 100% of [ACI→WF] chimeric hosts die from lethal GVHD after transplantation of unmanipulated donor (ACI) limbs. GVHD was associated with destabilization of the chimeric state. The purpose of this study was to eliminate GVHD in chimeric hosts after limb transplantation by pretreatment of the limb with irradiation while preserving stability of the chimeric state and donor-specific tolerance.

MATERIALS AND METHODS

Animals: Male (5- to 7-week old) ACI (RT1A^b) and Wistar Furth (WF, RT1A^a) rats weighing between 200 and 350 g were used. Animals were housed in a pathogen-free facility and were fed standard rat chow and given water ad libitum. All handling of animals was performed in accordance with the institutional Animal Care and Use Committee guidelines at the authors' American Association of Laboratory Animal Care-approved Research and Resource Center, School of Medicine, University of Louisville.

Groups: In group 1 (controls, n=6), ACI limbs were transplanted into untreated naive WF rats. In groups 2, 3, 4, and 5, [ACI→WF] chimeras were prepared by irradiation of WF rats with 950 cGy total body irradiation (TBI) and reconstitution with ACI rat BM (depleted of $\alpha\beta$ and $\gamma\delta$ T-cell receptor [TCR]-positive T cells). Limb transplantation was performed more than 28 days after BM reconstitution. In group 2 (controls, n=4), WF limbs were transplanted into [ACI→WF] chimeras. In group 3 (controls, n=4), "third-party" Fisher limbs were transplanted into [ACI→WF] chimeras. In group 4 (n=10), nonirradiated ACI limbs were transplanted into [ACI→WF] chimeras. In group 5 (n=8), irradiated (1,050 cGy) ACI limbs were transplanted into [ACI→WF] chimeras.

T-cell depletion of BM in vitro: ACI rat BM was harvested under aseptic conditions from femoral and tibial bones taken from ACI rats (flushing with Medium 199 (Life Technologies, Rockville, MD) containing 10 $\mu\text{g}/\text{mL}$ of gentamycin using a 22-gauge needle). Cell counts were adjusted to approximately 200×10^6 unseparated cells per donor animal before T-cell depletion (TCD). Cells were incubated with purified anti- $\alpha\beta$ and $\gamma\delta$ monoclonal antibodies (mAb) (mouse immunoglobulin [Ig] G; PharMingen, San Diego, CA) for 30 min at 4°C. BM cells were incubated for 60 min at 4°C with immunomagnetic beads at a bead-to-T-cell ratio of 20:1 and placed in a magnetic cell separator for 2 min to negatively select T cells. BM cells were washed, counted, and resuspended in Medium 199 plus gentamycin at a concentration of 100×10^6 BM cells/mL.

Verification of bead depletion using flow cytometry: To confirm the adequacy of TCD, aliquots of BM cells were set aside for flow cytometric analysis before bead depletion, after incubation with primary mAb to confirm coating, and after final depletion. Cells were incubated with either anti- $\alpha\beta$ TCR-fluorescein isothiocyanate (FITC) (R73; mouse IgG₁; BD

PharMingen), anti- $\gamma\delta$ TCR-FITC (V65; mouse IgG₁; BD PharMingen), or rat adsorbed goat antimouse IgG-FITC (BD PharMingen) for 30 min. After two washes, flow cytometric analyses were performed on FACS Calibur (Becton Dickinson, Bedford, MA). The gate chosen for selection of T cells included the upper 10% of cells by fluorescence in the predepletion BM and was kept constant for postdepletion samples.

Preparation of mixed allogeneic chimeras [ACI→WF]: Mixed allogeneic chimeras were prepared according to the authors' previously established protocol¹⁶. Briefly, WF hosts were conditioned with an unfractionated sublethal dose of 950 cGy of TBI. Using sterile technique, irradiated hosts were reconstituted within 4 to 6 hr of TBI, with 100×10^6 ACI rat BM cells (diluted in 1 ml modified Eagle's medium) through penile vein infusion.

Characterization of chimerism after BM reconstitution and limb transplantation: Flow cytometry on peripheral blood leukocytes (PBL) was used to assess engraftment of allogeneic BM 30, 60, and 90 days after BM reconstitution and chimerism 15, 30, 60, 120, and 150 days after limb transplantation. Antibodies against rat major histocompatibility complex (MHC) class I antigens were used to identify the percentage of PBL bearing ACI or WF rat MHC class I antigens. Whole blood was collected in heparinized plastic vials and aliquots of 100 μ L were stained with purified anti-RT1A^u (NR3/31; secondary label rat FITC IgG_{2a}; Serotec, Inc., Raleigh, NC) and FITC-labeled anti-RT1A^{ab} (C3; LOU/Cn IgG_{2b}; PharMingen) antibodies. The threshold for detection of donor cells was 0.5%. In all animals, engraftment was assessed. Chimeric hosts were typed for durable multilineage chimerism 30 and 90 days after BM reconstitution, using lineage markers for T cells ($\alpha\beta$ TCR-perCP: R73; rat IgG₁; Serotec), B cells (CD45RA-RPE: OX33, rat IgG₁; PharMingen), and macrophages (CD11b-RPE: OX42; rat IgG_{2a}; Serotec). Multilineage typing was also performed after limb transplantation at 150 days to assess durable engraftment and during clinical GVHD to detect alterations in cell lineage distribution coinciding with changes in levels of chimerism.

Irradiation of donor limbs: ACI donors were treated with 1,050 cGy of TBI. Hind limbs were procured from these animals soon after TBI and prepared for transplantation.

Hind-limb transplantation: Donor (ACI) and host (WF) animals were anesthetized with pentobarbital 60 mg/kg I.P.

Donor operation: The skin was incised proximal to the midhigh area; the femoral artery, vein, and nerve were dissected; and the individual muscle groups were divided proximally. The femur was divided at the midshaft. The limb was flushed for 10 min with heparinized Ringer's lactate.

Host operation: The bone was fixed with an intramedullary Kirschner wire (0.5 mm). Femoral vessels and nerves were anastomosed using microsurgical technique (10-0 nylon). The muscles and tendons were approximated using 5-0 nylon and the skin was closed using absorbable suture (5-0 Monocryl; Ethicon, Sommerville, NJ).

Clinical and histopathologic assessment for rejection and GVHD: Animals were monitored daily for signs of acute rejection of the limb or GVHD. Important clinical signs of rejection included edema, erythema, escharification, and necrosis. Necrosis was considered confirmation of frank rejection. GVHD was clinically confirmed by a syndrome of progressive weight loss; nasal discharge; erythematous rash over skin, paws, and ears; scruffy appearance with hair loss; and diarrhea¹⁷. All animals were weighed and assessed visually for signs of rejection and GVHD every day for the first month and weekly thereafter. To histologically assess or confirm rejection, skin and muscle biopsy specimens were taken from the CTA limb at 14 and 28 days and monthly thereafter or when clinical signs were present. To histologically assess or confirm GVHD, ear wedge skin biopsy specimens were taken monthly or when clinical signs were present. Animals were killed when rejection had caused necrosis of the transplanted limb or when GVHD had led to a weight loss of approximately 20%. In all other cases, animals were killed at 150 days. At the time the animals were killed, tissues including tongue, ear, liver, small intestine from the host, and skin and muscle from the transplanted limb and the host were harvested and fixed in 10% buffered formalin for hematoxylin-eosin staining.

Assessment of tolerance: Tolerance was assessed in vitro using mixed lymphocyte reaction (MLR) assays at the time the animals were killed. Spleens were harvested, diced, and crushed with a glass stopper to release lymphocytes. Isolated lymphocytes were ACK-lysed, washed, and resuspended in cMLR medium. Cultures were incubated at 37°C in 5% carbon dioxide pulsed on the fourth day with 1 μ Ci [³H]-thymidine (Perkin Elmer, Boston, MA), harvested on the fifth day with an automated harvester (PHD

Cell Harvester, Technology, Inc., Cambridge, MA) and counted in a β -scintillation counter (Beckman, Palo Alto, CA). Results were expressed as counts per minute (CPM) \pm SEM and as stimulation index (SI). The SI is the ratio of CPM generated in response to a given stimulator over baseline CPM generated in response to the host. Tolerance was assessed in vivo by transplanting donor (ACI) limbs to [ACI \rightarrow WF] chimeras (groups 4 and 5). Donor specificity was confirmed by transplantation of third-party Fisher rat limbs (group 3).

Statistical analysis: In all experiments, graft survival times between groups were calculated and compared according to the Kaplan-Meier method. Continuous variables were expressed as mean \pm SEM using analysis of variance and the post hoc Tukey's test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Hosts of donor BM depleted of $\alpha\beta$ and $\gamma\delta$ TCR-positive T cells engraft and do not exhibit GVHD: None of the [ACI \rightarrow WF] chimeras prepared by infusion of $\alpha\beta$ - $\gamma\delta$ T-cell-depleted ACI rat BM developed any signs of acute GVHD before limb transplantation. The T-cell content of donor BM was enumerated pre- and postdepletion with immunomagnetic beads tagged to monoclonal antibodies against the $\alpha\beta$ and $\gamma\delta$ TCR (Fig. 1). The actual total T-cell population in the BM of a rat varies from 3% to 6%. Bead depletion resulted in a reduction of $\alpha\beta$ TCR T cells from 1.34% \pm 0.08% to 0.05% \pm 0.01% and $\gamma\delta$ TCR T cells from 1.00% \pm 0.48% to 0.05% \pm 0.03%.

Evidence of stable multilineage chimerism before limb transplantation: All animals in groups 2, 3, 4, and 5 demonstrated a stable chimeric state before limb transplantation (at 30, 60, and 90 days after BM reconstitution). The results of one-color fluorescence-activated cell sorter analysis using FITC-labeled monoclonal antibodies in a representative chimera are shown at 30 days after BM reconstitution (Fig. 2). Determination of durable multilineage engraftment in the chimeras was performed before limb transplantation (at 30 and 90 days after BM reconstitution). Figure 3 shows multilineage typing at 30 days in a representative chimera. The percentage of donor-derived cells in chimeras varied between lineages, with T cells ranging from 22.8% to 42.3%, B cells ranging from 52.0% to 75.0%, and macrophages ranging from 35.3% to 62%.

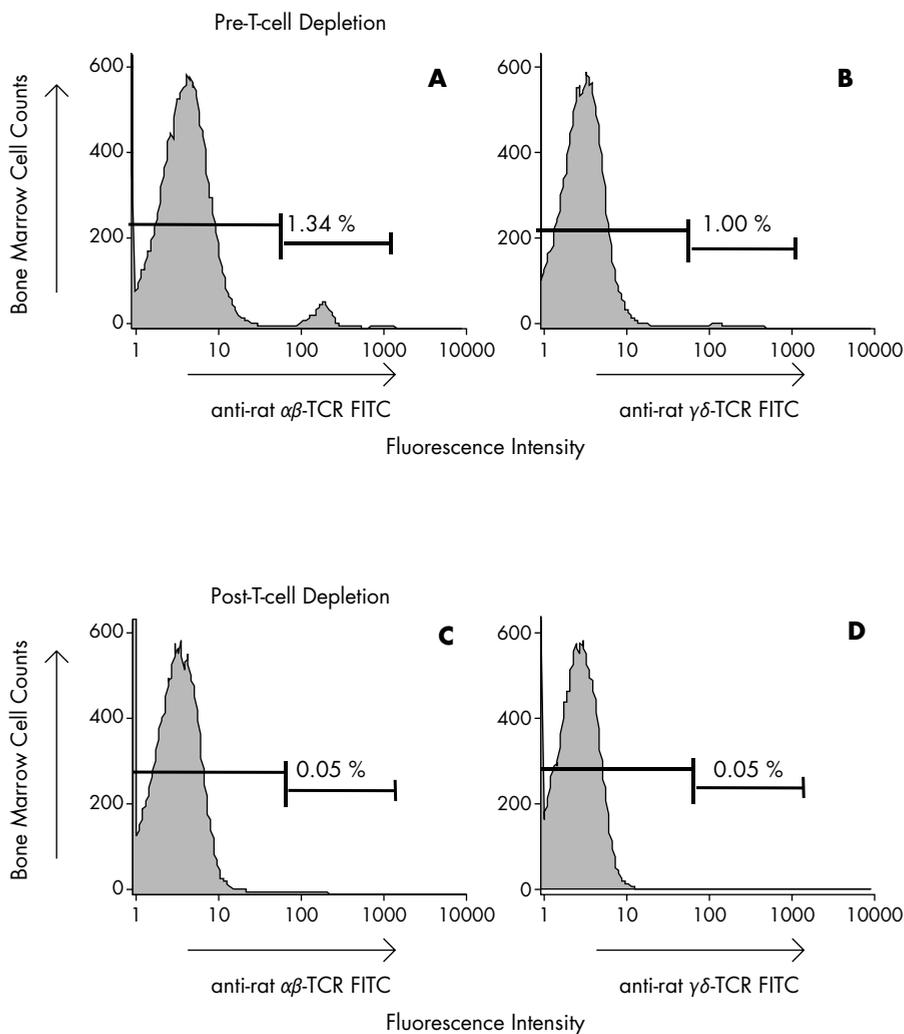


Figure 1. T-cell depletion of donor bone marrow. Histograms show adequacy of depletion of $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells using rat anti-adsorbed goat antimouse Ig monoclonal antibodies. (A and C) Pre- and post-TCD results of donor BM using anti-rat $\alpha\beta$ TCR-FITC. (B and D) Pre- and post-TCD results using anti-rat $\gamma\delta$ TCR-FITC. Histograms reveal that immunomagnetic bead depletion resulted in an excellent reduction of $\alpha\beta$ TCR T cells from $1.34\% \pm 0.08\%$ (A) to $0.05\% \pm 0.01\%$ (C) and $\gamma\delta$ TCR T cells from $1.00\% \pm 0.48\%$ (B) to $0.05\% \pm 0.03\%$ (D). Postdepletion was less than the isotype control (not shown). The gate chosen for selection of T cells includes the upper 10% of cells by fluorescence in the predepletion BM and was kept constant for postdepletion samples.

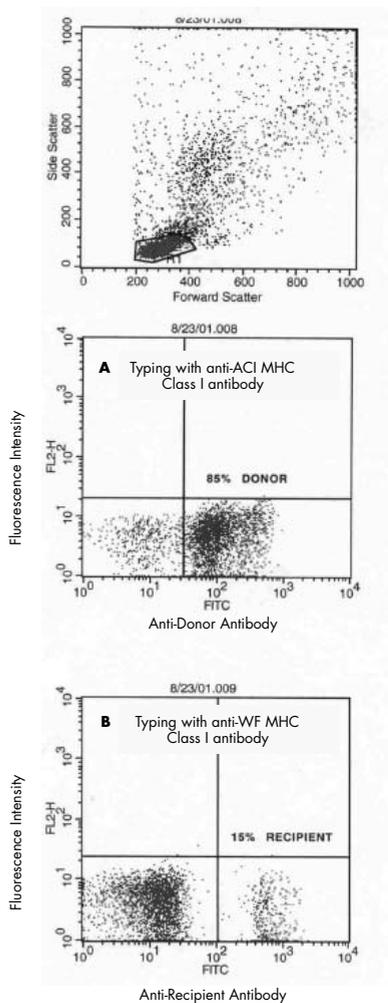


Figure 2. Confirmation of stable chimerism before limb transplantation. Dot plots show results of one-color fluorescence-activated cell sorter analysis in one representative chimera before limb transplantation. The mAb used to stain the cells are directed against the class I MHC antigen on the surface of donor (RT1A^b) or host (RT1A^u) cells. (A) Results of typing with mAb specific for the donor MHC class I antigen (RT1A^{ab}) and (B) results of typing with mAb specific for the host MHC class I antigen (RT1A^u).

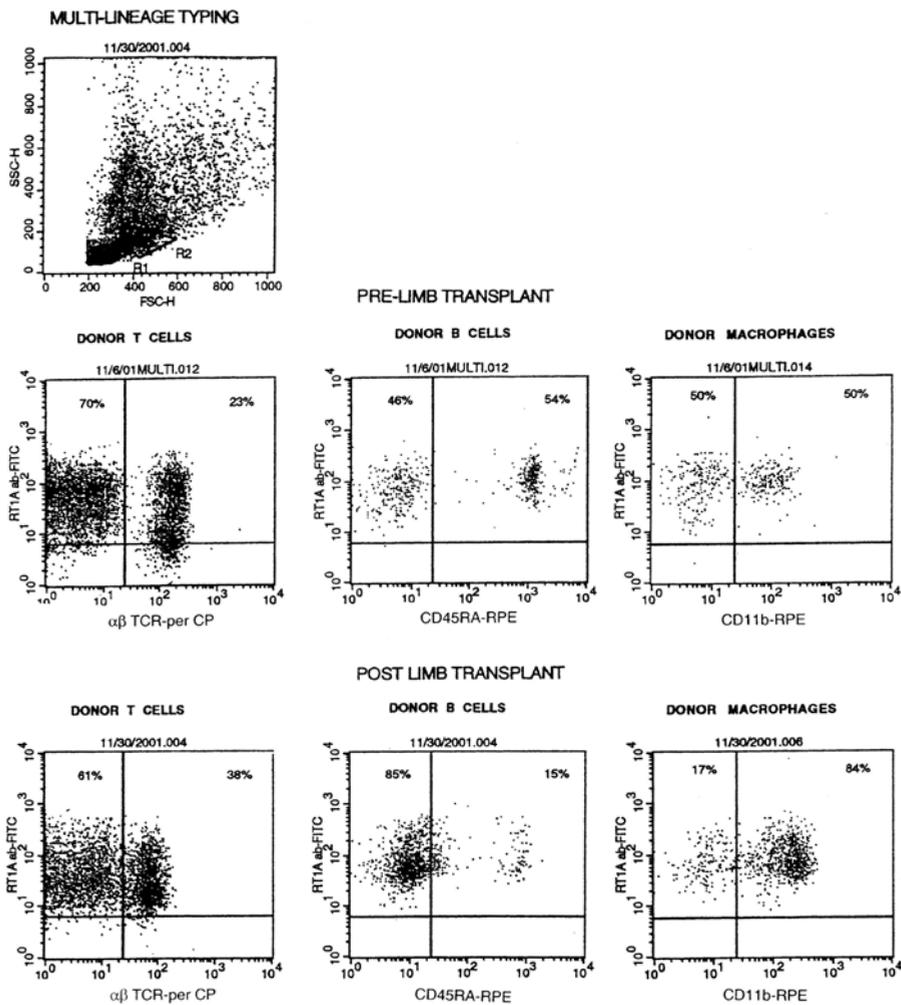


Figure 3. Multilineage analysis of donor chimerism before and after limb transplantation. Dot plots show results of fluorescence-activated cell sorter analysis used to enumerate the proportion of donor-derived lymphoid and myeloid lineages in mixed allogeneic chimeras [ACI→WF]. The specific lineage used for detection of T cells, B cells, and macrophages-granulocytes were $\alpha\beta$ TCR, CD45RA, and CD11b, respectively.

Upper. Pre-limb transplant multilineage typing of a representative chimera (group 4) performed at 30 days after BM reconstitution. The percentage of donor-derived cells in each lineage is as follows: $\alpha\beta$ TCR T cells, 23%; B lymphocytes, 54%; and donor macrophages, 50%.

Lower. Post-limb transplant multilineage typing from the same animal performed at onset of GVHD. The percentage of donor-derived cells in each lineage is as follows: $\alpha\beta$ TCR T cells, 38%; B lymphocytes, 15%; and donor macrophages, 84%.

Effect of limb transplantation on donor chimerism levels and clinical outcome:

Chimeras were followed for percentage levels of donor chimerism after limb placement (Fig. 4). After hind-limb transplantation, hosts that died from GVHD (group 4) demonstrated a greater increase (approximately 18%) of donor chimerism than those that did not (approximately 3% in group 5). A significant increase in donor (ACI) chimerism was uniformly associated with acute GVHD. Flow typing on PBL and BM (of transplanted and contralateral limbs) of hosts at the time the animals were killed revealed donor chimerism levels that were remarkably similar in both PBL and BM. Chimeric WF hosts (group 2) receiving nonirradiated limbs from strain-matched WF donors demonstrated a significant decrease in ACI chimerism level ($5.0\% \pm 4.3\%$) after limb transplantation (when compared

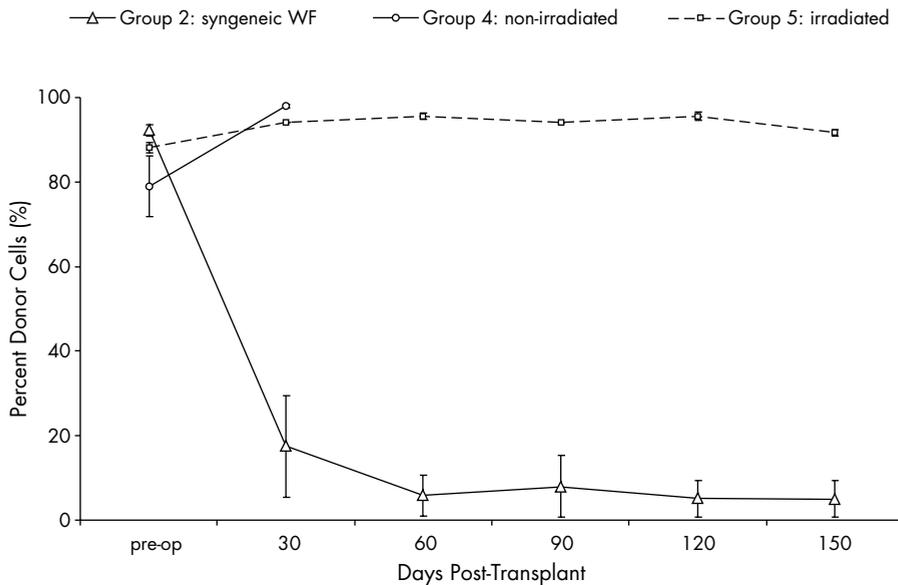
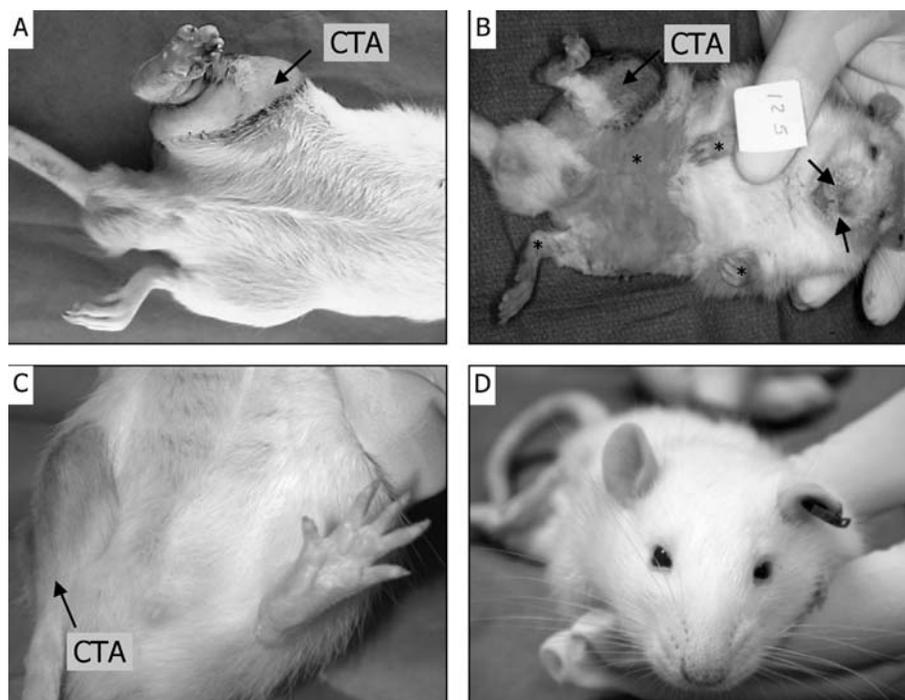


Figure 4. Levels of donor chimerism after limb transplantation. The levels (mean \pm SEM) of donor chimerism after limb transplantation of group 2 (triangles) are compared with levels in groups 4 (circles) and 5 (squares). The mean levels of donor chimerism in [ACI \rightarrow WF] hosts before limb transplantation were in the range of 75% to 92%. Note that in group 4 there is an acute increase in the donor chimerism before 30 days after ACI limb transplantation. Clinically, this increase culminated in lethal GVHD. Comparison of levels of donor chimerism is shown at 60, 90, 120, and 150 days after limb transplantation in groups 2 and 5. Note that long-standing survivors in group 5 demonstrated stable chimerism that was consistent with a tolerant state toward the ACI donor limb. In group 2, transplantation of WF limbs led to a sharp decrease in donor chimerism, but tolerance was maintained.

with pretransplant ACI levels [$85.5\% \pm 1.2\%$]). However, this was not associated with acute rejection or acute GVHD. Multilineage typing (Figure 3) showed that the donor cell pool of $\alpha\beta$ TCR T cells and the neutrophil-macrophage population increased in the peripheral blood after limb transplantation and the donor B-lymphocyte population diminished in the peripheral blood after limb transplantation.

Irradiation of donor limbs before transplantation prevents GVHD in chimeric hosts: All animals from group 4 that received nonirradiated donor-specific ACI limbs died from histologically proven acute GVHD, whereas those that received irradiated ACI limbs (group 5) did not. The clinical appearance of animals in groups 4 and 5 is compared with that of group 1 (controls) in Figure 5. Pre- and posttransplant body weight of rats with acute GVHD (group 4) is compared with weight data from other groups in Figure 5. The degree of weight loss in group 4 was approximately 30% in the first 3 weeks after limb transplantation ($P < 0.05$) when compared with groups 2 and 5. All animals in group 4 showed typical histologic signs of severe GVHD (Figs. 5 and 6). In group 5, absence of acute or chronic GVHD was confirmed histologically 150 days after transplantation (Figs. 5 and 6) by the absence of (1) dermal sclerosis, periecrine infiltration, or fibrosis of hypodermis in the skin; (2) signs of mucosal



inflammation with destruction of exocrine glands or sclerosis-strictures in the esophagus; or (3) hyalinization of portal triads, fibrotic obliteration of bile ducts, or cholestasis in the liver.

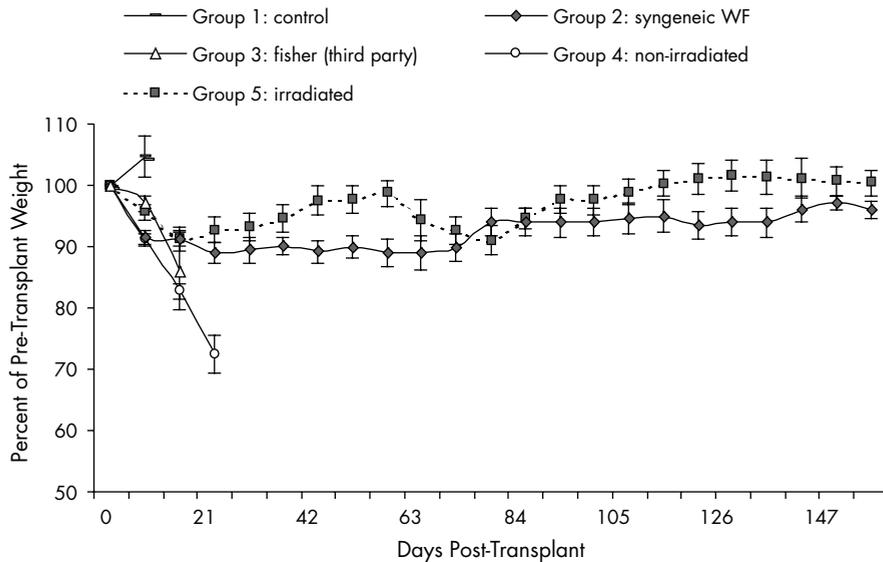


Figure 5. Clinical assessment for GVHD and rejection after limb transplantation.

Previous page. (A) Rejection of limb transplants by controls in group 1. Note edema, erythema, escharification, and necrosis of the transplanted limb. Peeling off of skin on minimal pressure was considered confirmation of frank rejection. (B) Lethal GVHD in group 4. Note the characteristic signs of acute GVHD including erythema of the skin over the abdomen, dermatitis of ears, diffuse hair loss, scruffiness, nasal discharge, and general moribund appearance of the animal. (C and D) Animals in group 5 showing prolonged survival without evidence of GVHD after transplantation of an irradiated limb. Note the marked contrast in clinical appearance without any signs of GVHD or rejection.

Above. Weight loss (mean±SEM) was used as an important prognostic determinant of acute GVHD in transplanted chimeras. Controls in group 1 (dashes) rejected their limbs in 5.7 ± 1.5 days. No weight loss was noted during this period. Chimeras that underwent transplantation with WF limbs in group 2 (diamonds) demonstrated a 10% to 15% weight loss in the first 2 weeks after limb transplantation, after which the body weight remained stable for 2 months and then started to increase until the endpoint of the study. Chimeras that underwent transplantation with third-party Fisher rat limbs in group 3 (triangles) rejected their limbs in 7.3 ± 1.5 days, before which they demonstrated weight loss of approximately 15%. Chimeras that underwent transplantation with irradiated ACI limbs in group 5 (squares) demonstrated a similar acute weight loss not exceeding 10% in the immediate 2 weeks after limb transplantation, followed

by rapid improvement in weight gain. In all the above groups, the acute initial weight loss that was followed by gradual increase in body weight, which stabilized by day 150 (endpoint of study), was related to the acute catabolic state after hind-limb transplantation surgery. In contrast, in group 4 (circles), chimeras that underwent transplantation with nonirradiated limbs demonstrated greater than 20% weight loss in the first 2 to 3 weeks after limb transplantation that culminated in death of 100% of animals by day 28.

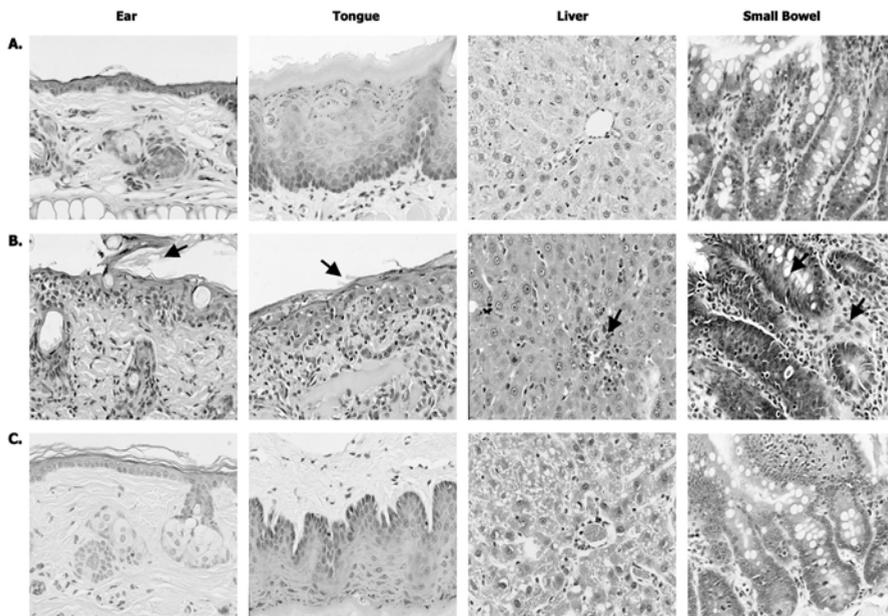


Figure 6. Histopathologic assessment of GVHD. Hematoxylin-eosin–stained sections (x 400) from group 2 (A) are compared with those from group 4 (B) and group 5 (C). (Ear [skin]) Group 4: Note perivascular lymphocytic infiltrate, dyskeratosis, and liquefaction of epidermis with bulla (arrow) formation and dermal edema. Groups 2 and 5: No abnormalities noted. (Tongue) Group 4: Note marked lymphocytic exocytosis, vacuolization of basal cells, and edema of muscularis with denudation of squamous epithelium (arrow). Groups 2 and 5: No abnormalities noted. (Liver) Group 4: Evidence of periportal lymphoid infiltration (arrow) with hepatocyte and bile duct injury. Small hyperchromatic lymphocytes with pyknotic nuclei are also seen. Groups 2 and 5: There is some infiltration with small lymphocytes around portal triads without associated liver cell or bile duct injury. (Small bowel) Group 4: Note moderate villous atrophy (arrow) with variable degree of flattening and numerous crypt mitoses (arrow) with crypt hyperplasia. Groups 2 and 5: Other than a slight decrease in the numbers of plasma cells and small lymphocytes in the lamina propria, the mucosal histology is normal.

Chimeras receiving limb transplants exhibit donor-specific tolerance in vitro and in vivo: Splenocytes from [ACI→WF] chimeras transplanted with irradiated ACI limbs (group 5) showed hyporesponsiveness toward donor (ACI) with intact and significant reactivity toward third-party (Fisher) rat splenocytes ($P < 0.05$). The proliferation responses (expressed as $\text{CPM} \pm \text{SEM}$) from the MLR assays performed in the various groups are summarized in Figure 7. Tolerance and immunocompetence were confirmed in vivo by the prolonged survival of donor-specific ACI hind-limb (group 5) transplants and vigorous rejection of third-party Fisher limbs (group 3) by [ACI→WF] hosts. A Kaplan-Meier life table of survival statistics is shown in Figure 8. Absence of acute and chronic rejection in group 5 and acute rejection in group 3 were also confirmed histologically. None of the long-surviving chimeras in group 5 developed signs of chronic rejection including intimal hyperplasia or medial vascular sclerosis culminating in vasculopathic graft failure.

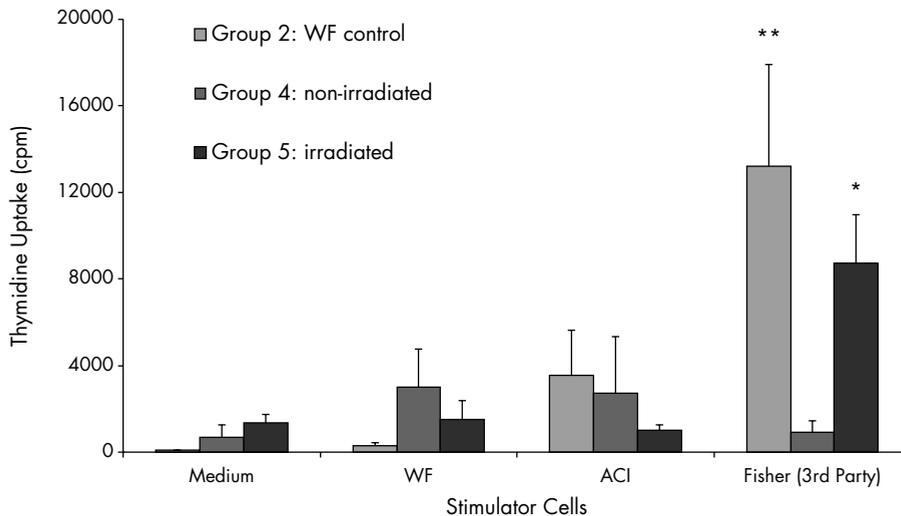


Figure 7. MLR assay. Results of one-way MLR assay are shown for naive WF controls (light grey bars), and animals from groups 4 (dark grey bars) and 5 (black bars). The persistent, severe immunoincompetence in chimeric hosts dying from GVHD after receiving nonirradiated ACI limbs in group 4 is reflected in depressed reactivity toward both donor specific (ACI) and third-party (Fisher rat) antigens. Chimeras receiving irradiated ACI limbs in group 5 demonstrate excellent reactivity toward third-party (Fisher rat) antigens with donor-specific (ACI) hyporesponsiveness. Results from both these groups are compared with WF controls. Also, the baseline counts in cMLR medium (medium on graph) are also shown for comparison. Results are expressed as

CPM+SEM. *Significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward ACI ($P \leq 0.01$) and WF cells ($P \leq 0.05$) in group 4. **Significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward WF cells ($P \leq 0.01$) in controls.

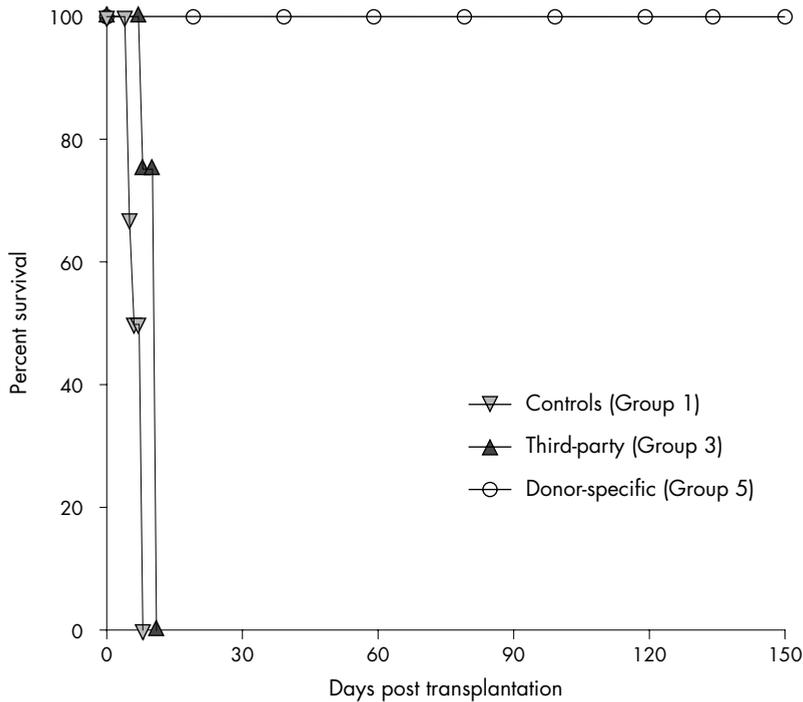


Figure 8. Survival of limb transplants is shown using the Kaplan-Meier life table. Donor-specific (ACI) or third-party (Fisher rat) limbs were transplanted 4 to 6 months after BM reconstitution into stable chimeras. ACI limbs were transplanted into naive WF hosts that served as controls. (Inverted triangles) Group 1 (controls) rejected their limbs in 5.7 ± 1.5 days. (Triangles) Chimeras that underwent transplantation with third-party limbs (group 3) rejected them in 7.3 ± 1.5 days. (Circles) Chimeras that underwent transplantation with donor ACI limbs (group 5) demonstrated prolonged survival of limbs (158 ± 2.3 days) until the endpoint of the study. Results from group 3 confirm intact third-party responses and excellent immunocompetence in [ACI→WF] chimeras after limb transplantation. Results from group 5 indicate the donor-specific hyporesponsiveness of [ACI→WF] chimeras after limb transplantation. Graft survival was determined by daily visual examination for signs or symptoms of rejection. Percentage survival of donor-specific grafts was 100% at the endpoint of the study. Rejection of Fisher rat grafts was brisk and comparable to controls.

DISCUSSION

CTA are composed of multiple tissues, some of which are highly immunogenic and therefore induce rapid rejection¹⁸ and others that can initiate GVHD in the host^{13,15}. The authors chose the rat hind-limb CTA model for this study because it provides a stringent model for testing tolerance induced by MAC. The rat model, unlike the mouse, is better suited for study of GVHD because GVHD is more easily induced in the rat and closely resembles the human syndrome¹⁹. The rat hind limb represents some of the key tissues (skin, muscle, vessel, nerve, lymph nodes, bone, and BM) that would be included in many clinical CTA (hand or head and neck transplants). Similarly, certain components of clinical CTA such as lymph nodes and BM can, in addition to causing rejection, initiate lethal GVHD because of their mature T-cell content²⁰.

Historically, HSC chimerism has been established in several experimental models including rodents^{16,21}, large animals and primates^{22,23}, and most recently humans²⁴. After the first report of clinical tolerance to donor skin allografts in recipients of donor BM²⁵, drug-free acceptance of renal allografts (in patients undergoing BM transplantation for hematopoietic malignancy and later developing renal failure) has been reported in humans²⁶. Hewitt et al. pioneered studies examining the role of BM within transplanted rat hind limbs in induction of chimerism and tolerance or potential development of GVHD. In one study, they demonstrated that when parental Lewis rat limbs were transplanted into LBN F1 hybrid hosts, GVHD developed in approximately 35% to 40% of recipients²⁷.

Recent studies report that established chimeras previously made tolerant to donor-specific antigens are susceptible to GVHD after allograft transplantation. In a rat solid-organ transplant model, Morrissey et al.²⁸, demonstrated that ACI small-bowel transplants into [ACI→Lewis] chimeras results in susceptibility of 100% (six of six) of these tolerant hosts to small-bowel-induced lethal GVHD. In a CTA model, Foster et al.¹⁵ demonstrated for the first time that ACI hind-limb transplants into [ACI→WF] chimeras results in susceptibility of these tolerant hosts to CTA-induced GVHD. MAC was induced by infusion of $\alpha\beta$ TCR-positive (TCR⁺) depleted syngeneic (WF) and allogeneic (ACI) BM into WF hosts after irradiation, antilymphocyte serum, and FK506 peritransplant immunosuppression. In the present study, the authors performed rat hind-limb transplants across a similar donor-host combination with similar manipulation of CTA and similar duration of follow-up. A similar dose of donor BM cells was used to prepare mixed chimeras. The authors' study was different from that by Foster et al. in that only radiation-based conditioning was used and only Allogeneic (donor ACI) BM deplet-

ed of both $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells was used to establish MAC in WF recipients. Comparisons of results from the authors' study to those of Foster et al. in comparable experimental groups revealed key differences in outcome. The immunocompetent cells causing GVHD are T cells present in the BM²⁹ or lymph nodes in the allograft, and the severity of GVHD-related mortality increases with the logarithm of the number of T cells in the graft³⁰. Foster et al. hypothesized that the incidence of GVHD in their study, even though low, was related to the transplantation of intact BM with the hind limb. In contrast, the authors of the present study hypothesized that because lymph nodes contain 40% to 75% of the T cells³¹, and because 95% of mature T cells in the lymphatic compartment express the $\alpha\beta$ TCR, the potential risk of severe GVHD was greater with transplantation of lymph nodes and not BM^{32,33}. The authors confirmed this hypothesis in another study in the same model by demonstrating that transplantation of limbs after surgical removal of lymph nodes from CTA (otherwise containing intact BM) prevented GVHD in chimeric hosts³⁴.

The authors previously reported that TCD of both $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells from donor BM before transplantation resulted in 100% engraftment and prevented GVHD³⁵. The authors believe that this was because the BM population that facilitates stem-cell engraftment is $\alpha\beta$ and $\gamma\delta$ TCR-negative and their technique of TCD of ACI marrow did not remove this population³⁶. In this study, transplantation of nonmanipulated ACI limbs containing nontolerant mature donor lymphoid cells to established [ACI→WF] chimeras (group 4) initiated classic symptoms and signs of acute and lethal GVHD in 100% of chimeric hosts. In contrast, Foster et al. reported that only one of nine chimeric hosts (11%) that underwent transplantation with nonirradiated ACI limbs developed GVHD in their study. The authors of the present study also found that the levels of donor chimerism increased significantly after limb transplantation in 100% of animals dying from acute GVHD (group 4). Several underlying mechanisms may be responsible for the "destabilization" of the previously stable MAC in the authors' hosts. First, an influx of donor HSC from the BM compartment of the CTA could increase the level of donor chimerism in the host but not attack the host to cause GVHD. The occurrence of GVHD in all animals in group 4 refutes this hypothesis. Second, an expansion of mature alloreactive cell lineages from the donor leg could increase the level of chimerism in the host and cause GVHD, but such an increase is not reflected in the BM or other central hematopoietic tissues. The authors performed flow typing on both PBL and BM of hosts at the time the animals were killed. The level of donor chimerism was remarkably similar in both PBL and BM. This finding therefore refutes

this hypothesis. Finally, immunocompetent but nontolerant T cells transplanted with the limb may attack host HSC and mature lineages. This could result in an increase in the level of donor chimerism and GVHD. Furthermore, such an increase will be reflected in the host BM. This is the reason for the BM aplasia that results from transfusion-induced GVHD. The authors' results of multilineage typing for donor cell lineages ($\alpha\beta$ TCR⁺ T cells, B cells, and macrophages) before and after (Fig. 1) limb transplantation confirmed this as the mechanism underlying change in MAC in their model. The authors found a significant increase in donor $\alpha\beta$ TCR⁺ T cells in the host PBL after limb transplantation that clinically correlated with a state of lethal GVHD. Because the BM used to prepare the chimeras was depleted of $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells, the authors can postulate that the mature donor $\alpha\beta$ TCR⁺ T-cell pool that increased after limb transplantation was derived from the limb.

In the authors' study, significant weight loss was a direct correlate of mortality in acute GVHD (group 4). The authors analyzed samples of skin (ear), tongue, small bowel, and liver, because these are the principal target organs of acute GVHD apart from the immune system. Selective epithelial damage in target tissues was the hallmark of GVHD in animals dying from acute GVHD (group 4). In this model, GVHD resulted in maximal damage at the tips of the rete ridges in the skin, at the base of the crypts in the small bowel, and in the periductular epithelium in the liver. However, the authors found that tissues that were lined with squamous epithelium were not all injured in the same fashion. The most severe involvement was in the skin, followed by the tongue. The authors also found that histologic changes in the tongue were more sensitive indicators of the disease. The presence and severity of injury to internal target tissues was proportional to the extent of inflammatory injury in the tongue. There was a consistent correlation between involvement of the tongue with that of the liver in all animals in group 4. In contrast to the marked damage to liver, the authors found relatively mild involvement of small bowel by GVHD. Clinically affected animals that had diarrhea revealed minimal mucosal and crypt destruction without the mucosal ulceration that is characteristic of human GVHD.

Chimeric hosts that underwent transplantation with nonirradiated limbs from WF rats (syngeneic to the host) did not die from acute GVHD or rejection and showed prolonged survival (group 2). In the study by Foster et al., 100% of syngeneic WF hind limbs transplanted into hosts (donor chimerism range, 0%–19%) were acutely rejected¹⁵. They hypothesized that the number of donor cells influenced the incidence of rejection at the level of the limb. Interestingly, however, similar transplants (also in the same study) survived indefinitely in hosts at a donor chimerism level ranging from 69%

to 90%. In the present study, the authors also found a significant decrease in the level of donor chimerism (to <10%) after transplantation of the WF limb. Foster et al. do not report similar data in their study. The authors of the present study hypothesized that immunocompetent T cells from the transplanted WF limb could have attacked engrafted ACI stem cells in the [ACI→WF] host. This reaction of naive WF cells from the transplanted limb with the ACI rat HSC from the infused BM could have resulted in loss of donor chimerism in the host. Also, the authors found that levels of MAC in PBL and BM in both groups were similar, as demonstrated by flow cytometry. These results are similar to previously demonstrated studies that naive host CD4⁺ T cells infused into MAC hosts eliminate donor chimerism in a dose-dependent fashion³⁷.

Manipulation of BM³⁸ and solid-organ grafts³⁹ with radiation (gamma or ultraviolet) can successfully prevent GVHD. Despite this ability of irradiation to limit the ability of donor T cells to proliferate and cause GVHD, it does not have significant effect on cytotoxic activity and graft antigenicity⁴⁰. In group 5, the authors determined whether irradiation of highly antigenic donor limb CTA was a simple and practical approach to prevent GVHD in their model. In group 5, this approach prolonged survival of limb transplants associated with stable posttransplant levels of chimerism. This is important because declining levels of donor MAC may reflect poor HSC engraftment associated with loss of tolerance. The level of donor chimerism was similar in both PBL and in BM of the transplanted limbs, indicating that the pluripotent ACI stem cell had engrafted in the irradiated transplanted limbs. Results of in vivo assessment, in vitro assays, and clinical and histopathologic examination of tissue for signs of rejection or GVHD confirmed that all long-surviving chimeras in group 5 were tolerant while being free from GVHD.

The risks of both chronic rejection and chronic GVHD are directly proportional to the number of acute episodes of the respective condition. Clinical and histopathologic examination in group 5 after follow-up for 150 days after limb transplantation (an average of 11 months after BM reconstitution) revealed that tolerant animals did not develop clinical signs of chronic rejection as manifested by graft failure attributable to vasculopathy. [ACI→WF] chimeras were followed for periods of 4 to 6 months before limb transplantation to exclude chronic GVHD caused by the reconstituted BM. Furthermore, none of these animals receiving irradiated donor limbs showed signs of chronic GVHD at 150 days in typically affected target tissues such as skin, esophagus, and liver.

Together, these results indicate that irradiation of limb CTA (containing BM and lymph nodes) before transplantation is a simple and practical approach that consistently prevented GVHD and destabilization of MAC in chimeric hosts. Prolonged survival of chimeric hosts without evidence of both acute and chronic rejection and acute and chronic GVHD was achieved. The authors' long-term goal is to achieve donor-specific tolerance and avoid GVHD in human composite tissue allotransplantation using MAC. Composite tissue transplantation has become a clinical reality. As the mechanisms of chimerism induction and maintenance and the potential for graft-versus-host reactivity resulting from the complex tissue transplanted become better defined, strategies to improve clinical outcome will emerge.

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Chapter **7**

Composite tissue allotransplantation in chimeric hosts part II. A clinically relevant protocol to induce tolerance in a rat model

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INTRODUCTION

Composite tissue allografts (CTAs) hold tremendous potential in reconstruction of soft tissue and skeletal defects. Nevertheless, their routine use is prevented by the toxicity of the immunosuppressant drugs required to prevent rejection. Bone marrow transplantation (BMT)-induced hematopoietic stem cell (HSC) macrochimerism has been shown to effectively induce donor-specific tolerance¹ to a variety of allografts such as skin², heart³, lung⁴, and pancreatic islets⁵ in rodents, large animals⁶, and primates⁷, eliminating or minimizing the need for immunosuppressant drugs. In humans, BMT has been shown to confer acceptance of donor-specific skin⁸ and kidney allografts⁹ without immunosuppression.

Despite the promising potential of mixed allogeneic chimerism (MAC) in inducing tolerance to CTAs, important hurdles must be overcome before its clinical application. In the clinical setting, graft-versus-host disease (GVHD) because of the donor BMT¹⁰ and the toxicity of ablative host conditioning are considered to be the main hurdles standing in the way of using MAC-induced tolerance. In addition to these hurdles, the fact that a 28-day delay period is required between donor BMT and allograft transplantation in experimental MAC protocols¹¹ constitutes another important obstacle for its clinical application. This requires that chimerism be induced at least 28 days before allograft transplantation. Conventional experimental protocols for preparing chimeras involve a sequential course of steps: host conditioning, donor BMT, characterization of chimerism by flow cytometry (at 28 days), and the donor allograft transplantation. Despite this tedious protocol and the delay period required between induction and transplantation, this method of inducing tolerance using MAC has been successful in several rat transplant models^{12,13}.

Clinically, however, this delay period might not be as important in select cases of living solid-organ transplantation in which the donor organ is procured from living donors, allowing a delay between bone marrow (BM) infusion and organ transplantation; this would not be the case in CTA. In a CTA, such as a hand transplant, the hand is always procured from a cadaveric donor, therefore logistically the BM and the hand would have to be procured and infused or transplanted into or onto the host simultaneously. The delay period has been considered a requirement for engraftment and repopulation of donor BM cells in the host. It has also been believed that if allograft transplantation is performed before successful engraftment of donor BM has been achieved, it may interfere with the establishment of tolerance. Engraftment of donor BM is critical for survival of both the donor HSCs and of the conditioned host¹⁴, and it is dependent on both donor

and host factors. The degree of host conditioning, the presence of residual host immunocompetent cell populations, and the temporal relationship between host BM reconstitution and allograft transplantation are some of the important host variables. The last factor was most pertinent to our study.

We¹⁵ and others¹⁶ previously reported that mixed chimeras created with donor BM (depleted of both $\alpha\beta$ and $\gamma\delta$ T-cell receptor [TCR]⁺ T cells) showed 100% engraftment with no evidence of GVHD in a rat model. Our partial myeloablative conditioning protocol allowed for consistent MAC whereas preserving host immunocompetence in the (ACI→WF) chimeras. These mixed chimeric hosts were also tolerant toward donor-specific CTA, transplanted sequentially after the donor BMT¹⁷. In the present studies, we evaluated whether similar results could be achieved in conditioned hosts receiving a donor CTA placed simultaneously with the donor BMT.

CTAs (e.g., rat hind limb) are composed of a highly antigenic composite of multiple tissues. It has not been evaluated whether the placement of such highly antigenic allografts coincident with the donor BMT would be successful. For the first time, this study developed a protocol to induce tolerance to a CTA through MAC in a clinically relevant time frame using a rat hind limb transplant model.

MATERIALS AND METHODS

Animals: Male (5–7 week) ACI (RT1A^b) and Wistar Furth (WF, RT1A^u) rats weighing between 200 and 350 g were used. Animals were housed in a pathogen-free facility and were fed standard rat chow and given water ad libitum. The study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the “Guide for the Care and Use of Laboratory Animals” (Department of Health and Human Services, Publication No. [National Institutes of Health] 86–23).

Groups: Thirty-seven rats were allocated into five groups. In group 1 (n=6), host WF rats received donor ACI limb transplants without treatment (naïve controls). In groups 2 and 3, a conventional or “sequential” chimeric protocol was used (discussed next). In group 2 (n=4), host (ACI→WF) chimeric rats received limbs from Fisher donors (third-party controls). In group 3 (n=10), host (ACI→WF) chimeric rats received limbs from ACI donors (lethally irradiated with 1,050 cGy) 50 to 70 days after BMT. In groups 4 (n=7) and 5 (n=10), a “simultaneous” chimeric protocol was

evaluated (discussed next). The only difference between groups 4 and 5 was that host animals in group 4 received temporary immunosuppression with tacrolimus and mycophenolate mofetil (MMF) for 28 days.

T-cell depletion of donor bone marrow in vitro: An established method was used to perform T-cell depletion¹⁸. Briefly, BM was harvested under aseptic conditions from femoral and tibial bones taken from ACI donors (flushing was performed with Medium 199 [Life Technologies, Grand Island, NY] containing 10 µg/mL of gentamicin using a 22-gauge needle). Cell counts were adjusted to approximately 200×10^6 unseparated cells per donor animal before T-cell depletion (TCD). Cells were incubated with purified anti $\alpha\beta$ and $\gamma\delta$ monoclonal antibodies (MoAbs) (mouse immunoglobulin [Ig]G; Pharmingen, San Diego, CA) for 30 min at 4°C. BM cells were incubated for 60 min at 4°C with immunomagnetic beads at a bead to T-cell ratio of 20:1 and placed in a magnetic cell separator for 2 min to negatively select T cells. BM cells were washed, counted, and resuspended in Medium 199 plus gentamicin at a concentration of 100×10^6 BM cells per milliliter. To confirm the adequacy of T-cell depletion, aliquots of BM cells were set aside for flow cytometry analysis before bead depletion, after incubation with primary MoAb (to confirm coating with MoAb), and after final depletion. Cells were incubated with anti- $\alpha\beta$ TCR-fluorescein isothiocyanate (FITC) (R73; mouse IgG₁; Pharmingen), anti- $\gamma\delta$ TCR-FITC (V65; mouse IgG₁; Pharmingen), or rat adsorbed goat anti-mouse IgG-FITC (Pharmingen) for 30 min. After two washes, flow cytometry analyses were performed on a FACS Calibur (BD Biosciences, San Diego, CA).

Sequential protocol for mixed allogeneic chimerism: In groups 2 and 3, mixed allogeneic chimeras were prepared according to our established sequential protocol^{15,17}. Briefly, WF hosts were conditioned with 950 cGy of unfractionated total body irradiation (TBI). By using a sterile technique, irradiated hosts were reconstituted within 4 to 6 hr of TBI, with 100×10^6 of ACI rat BM cells (TCD and diluted in 1 mL of Dulbecco's minimum essential medium) by penile vein infusion. Engraftment of allogeneic BM was confirmed 4 weeks after BM reconstitution using flow cytometry to determine the percentage of peripheral blood lymphocytes (PBLs) bearing ACI or WF major histocompatibility complex (MHC) class I antigens. Whole blood was collected in heparinized plastic vials, and aliquots of 100 µL were stained with purified anti-RT1A^u (NR3/31; rat IgG_{2a}; Serotec, Oxford, UK) and biotinylated anti-RT1A^{ab} (C3; LOU/Cn IgG_{2b}; Pharmingen) MoAb for 30 min. Repeat flow typing was performed at 60 and 90 days after BM

reconstitution to confirm stable chimerism before limb transplantation. These host (ACI→WF) chimeric rats received donor-specific limbs from ACI donors (lethally irradiated with 1,050 cGy) or third-party limbs from Fisher donors, 50 to 70 days after BMT.

Simultaneous protocol for mixed allogeneic chimerism: In groups 4 and 5, a simultaneous protocol for induction of chimerism was evaluated. WF hosts conditioned with 950 cGy of unfractionated TBI received donor-specific limbs from ACI donors (lethally irradiated with 1,050 cGy), and within 4 to 6 hr they received 100×10^6 ACI rat BM cells (TCD and diluted in 1 mL of Dulbecco's minimum essential medium) by penile vein infusion.

Irradiation of donor limbs: Donor ACI rats were treated with 1,050 cGy of TBI before transplantation. We used our previously established protocol for pretreatment of donor limbs (using irradiation) to prevent the onset of GVHD^{15,17}.

Hind-limb transplantation: Animals were anesthetized with sodium pentobarbital 60 mg/kg intraperitoneally, and a sterile technique was used for all the surgical procedures. Both donor and host rats were simultaneously prepared for the limb transplantation by two microsurgeons. In the donor operation, the skin was incised proximal to the mid-thigh area, the femoral artery, vein, and nerve were dissected, and the individual muscle groups were divided proximally. The femur was divided at the mid-shaft. The limb was flushed for 10 min with heparinized lactated Ringers solution. In the host operation, the bone was fixed using a 0.5-mm Kirschner wire. Femoral vessels and nerves were anastomosed using microsurgical technique (10-0 nylon). The muscles and tendons were approximated using 5-0 nylon and the skin was closed using absorbable 5-0 Monocryl suture (Ethicon, Inc., Cincinnati, OH).

Immunosuppressive drug regimen: Rats in group 4 were treated using low doses of tacrolimus (Prograf, Fujisawa Healthcare Inc., Deerfield, IL) and MMF (Cell-CEPT, Roche Laboratories Inc, Nutley, NJ) in a combination therapy. The tacrolimus was diluted with 5% dextrose and administered intraperitoneally at 1 mg/kg daily during 14 consecutive days, starting with the day of surgery, followed by 1 mg/kg twice per week thereafter. MMF powder was reconstituted with saline solution and administered

orally (15 mg/kg daily). Animals were weaned from drug therapy 28 days after the BM and limb transplantation.

Characterization of chimerism after limb transplantation: Chimeras were characterized by flow cytometry of PBL after limb transplantation at 30, 60, 120, and 150 days to determine levels of donor chimerism.

Clinical and histopathologic assessment of rejection and Graft-Versus-Host Disease: Animals were monitored daily for signs of limb rejection or GVHD. Important clinical signs included edema, erythema, escharification, and necrosis. Peeling skin on minimal pressure was considered confirmation of frank rejection. All animals were weighed daily for 2 months and weekly thereafter. The primary clinical diagnosis of GVHD was based on previously described criteria^{19,20}. Using a 2-mm punch, skin and muscle biopsies from the CTA were taken at 1, 2, and 5 months. Target tissues for GVHD (including tongue, ear, liver, and small intestine) were harvested, fixed in 10% buffered formalin, and processed routinely for hematoxylin-eosin staining in a blind fashion.

In vitro assessment of tolerance: Mixed lymphocyte reaction (MLR) assays were performed at the end of the study or at the time of sacrifice. Spleens were harvested, diced, and crushed with a glass stopper to release lymphocytes. Isolated lymphocytes were lysed with ACK, washed, and resuspended in complete MLR medium. Cultures were incubated at 37°C in 5% CO₂ pulsed on the fourth day with 1 µCi [³H] thymidine (Perkin Elmer, Boston, MA), harvested on the fifth day with an automated harvester (PHD Cell Harvester, Technology Inc., Cambridge, MA), and counted in a beta scintillation counter (Beckman, Palo Alto, CA). Results were expressed as counts per minute (CPM) + standard error of mean (SEM) and as stimulation index. Stimulation index is the ratio of CPM generated in response to a given stimulator over baseline CPM generated in response to the host.

In vivo assessment of tolerance: Three to six months after BM reconstitution, (ACI→WF) chimeras were transplanted with nonirradiated ACI limbs to assess for donor specificity of tolerance. Confirmation of third-party reactivity was performed by transplantation of Fisher rat limbs to (ACI→WF) chimeras.

Criteria for euthanasia: Animals were killed when rejection caused necrosis of the transplanted limb or when GVHD led to a weight loss of approximately 20%. In all other cases, animals were killed at 150 days.

Statistical analysis: In all experiments, graft survival times between groups were calculated and compared according to the Kaplan Meier method. Continuous variables were expressed as mean \pm SEM using analysis of variance and the post hoc Tukey test. Differences were considered to be significant with a *P* value of less than 0.05.

RESULTS

Effects of T-cell depletion of bone marrow in vitro: None of the chimeras prepared by infusion of $\alpha\beta$ and $\gamma\delta$ TCD ACI rat BM developed any signs of GVHD before limb transplantation, and all demonstrated 100% engraftment.

Confirmation of stable chimerism after limb transplantation:

Flow cytometric analyses on PBL samples were obtained from all animals that survived to the end point of the study. The mean level of donor chimerism in eight of nine (ACI \rightarrow WF) chimeras before limb transplantation in group 3 was 88.1% \pm 1.2%. The level of chimerism after limb transplantation at 150 days posttransplantation was 92.3% \pm 0.8% (Fig. 1). Simultaneous donor-specific limb transplantation and BMT resulted in stable, high levels of donor mixed chimerism. The mean level of donor chimerism in three of seven (ACI \rightarrow WF) chimeras in group 4 and seven of ten (ACI \rightarrow WF) chimeras in group 5, at 30 days post-limb transplant and BMT, was 93.7% \pm 0.9% and 85.1% \pm 2.6%, respectively. High levels of donor chimerism (> 80%) were achieved and persisted stably throughout the study. At the time of sacrifice, the one surviving rat in group 4 exhibited 95% donor chimerism, whereas 6 of 10 rats in group 5 survived to the end of the study, and the mean level of donor chimerism was 93.3% \pm 0.5% (Fig. 1). Chimerism levels achieved with the simultaneous protocol in groups 4 and 5 correlated well with those achieved with the sequential protocol in group 3.

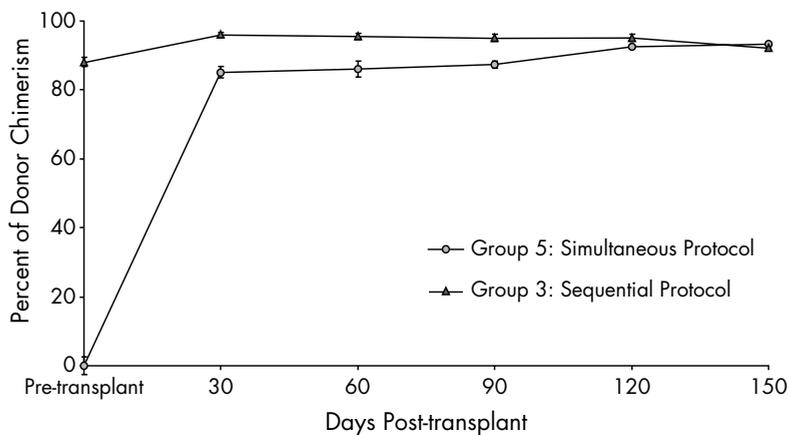


Figure 1. Levels of donor chimerism after limb transplantation in the sequential (triangle) and simultaneous (circle) protocols. Flow cytometry was performed on peripheral blood before operation and at 30, 60, 90, 120, and 150 days after limb transplantation. In group 5 (circle), the mean level of chimerism before limb transplantation in conditioned Wistar-Furth (WF) rats was 0%. Levels of donor chimerism indicate that by 30 days after conditioning, donor chimerism levels above the 80th percentile were achieved. This confirmed hematopoietic stem cell (HSC) engraftment. Despite early fluctuation, note that chimerism levels stabilized at high levels toward the end of the study.

Clinical and histopathologic confirmation of rejection and Graft-Versus-Host Disease after limb transplantation:

All animals maintained weight above 90% of their pretransplant weight at the end of the study. In group 1, the controls rejected their limbs in 5.7 ± 1.5 days. No weight loss was noted during this period. In group 2, the Fisher (thirdparty) rat limb was promptly rejected within 10 days. In group 3, one animal died at 33 days without clinical or histologic signs of rejection or GVHD, and the second animal was killed because of self-mutilation of the CTA limb at 1 day posttransplantation. Eight animals survived 150 days or more posttransplant. Although no clinical signs of GVHD were observed, skin and target tissue samples from two of these animals showed mild infiltration suggestive of subclinical GVHD. However, no histologic evidence of rejection was found in the transplanted limbs.

Weight loss was used as a reliable predictor for onset and progress of acute GVHD. Rats in groups 3, 4, and 5 demonstrated weight loss in the early postoperative period for up to 50 days but experienced rapid weight gain thereafter. The clinical appearance of an animal in the simultaneous protocol (group 5) is shown in Figure 2.



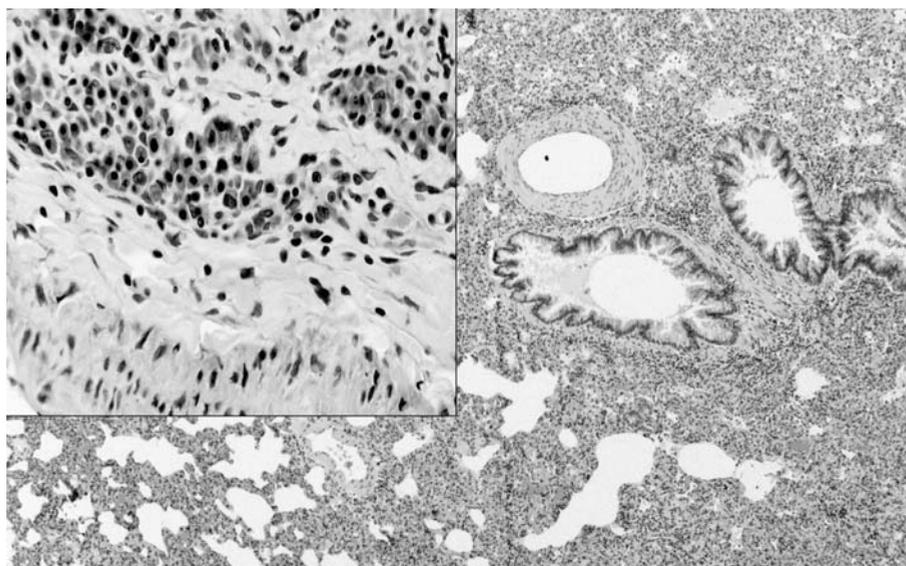
Figure 2. Clinical findings in group 5, with a representative animal (simultaneous protocol). Prolonged survival without evidence of graft-versus-host disease (GVHD) after transplantation of an irradiated ACI donor limb (arrow) transplanted simultaneously with donor bone marrow transplantation (BMT) were present in this group. Note that none of the characteristic signs of acute GVHD including erythema of the skin over the abdomen or diffuse hair loss and scruffiness were present. Note the healthy appearance of the ACI donor limb without evidence of rejection (arrow).

No clinical signs of rejection were observed in ACI limbs transplanted to (ACI→WF) chimeras simultaneously after BM reconstitution (groups 4 and 5). Histologic examination of skin (from CTA) and muscle biopsies performed at regular intervals during the experimental study confirmed the lack of rejection, further corroborating the tolerant state in these long-surviving chimeras.

In (ACI→WF) chimeras prepared using the simultaneous protocol in combination with immunosuppression therapy (group 4), five animals died before 60 days posttransplantation, four died between postoperative days 2 to 7, and one died on postoperative day 51. Analysis of peripheral blood in two animals revealed anemia and highly depleted cell counts. Red blood cells (RBCs) ($5.5 \times 10^6/\mu\text{L}$), white blood cells (WBCs) ($0.6 \times 10^3/\mu\text{L}$), and platelets ($3 \times 10^3/\mu\text{L}$) were comparable to normal values ($8 \times 10^6/\mu\text{L}$; $13.9 \times 10^3/\mu\text{L}$; $560 \times 10^3/\mu\text{L}$, respectively)²¹. No blood tests were possible in the other animals because they had died overnight. Flow typing in these two animals did not show evidence of donor chimerism.

Histology of target tissues and skin of the CTA harvested from these animals was negative for GVHD or rejection. One animal died on day 51, and the histology of the lungs revealed pneumonia but no evidence of rejection or GVHD (Fig. 3). One animal died at 132 days posttransplantation, and the histology revealed lymphoma in spleen, lymph nodes, and lungs, but there were no signs of rejection or GVHD (Fig. 3). Only one animal in this group survived to the end of the study (150 days posttransplant). The histology in this animal was negative for rejection (skin of CTA) or GVHD (solid organ/skin).

From the 10 (ACI→WF) chimeras prepared using the simultaneous protocol and conditioned with 950 cGy of TBI alone (group 5), four animals died at 7, 9, 25, and 42 days posttransplantation. In the animal that died at 7 days posttransplantation, the RBCs ($4 \times 10^6/\mu\text{L}$), WBCs ($4 \times 10^3/\mu\text{L}$), and platelets ($16 \times 10^3/\mu\text{L}$) were highly depleted. In the two other animals, blood tests were not possible because the animals were dead several hours before being noticed. However, no clinical or histologic signs of rejection were present in the transplanted limbs in the three animals. In the animal that died 42 days posttransplantation, blood tests were not possible, but histologic analysis showed signs of mild GVHD in skin and target solid organs (e.g., small bowel, tongue, and liver). Six animals completed the study (150 days posttransplantation) with no clinical or histologic signs of rejection or GVHD.



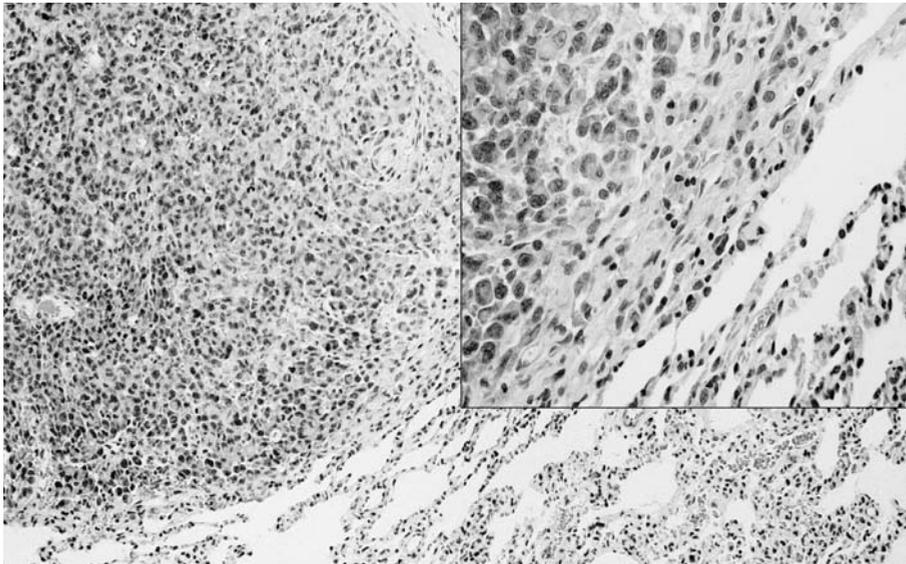


Figure 3. Histopathologic findings in group 4.

Left panel. Histopathologic specimen from an animal that died on day 51 posttransplant (simultaneous protocol with drugs). The blood cell counts demonstrated an increase of white blood cells (WBCs), and histology revealed pneumonia in the lungs with extensive interstitial infiltration by dense inflammatory cells (hematoxylin-eosin [H&E] stain, magnification x100). High power reveals the inflammatory cells as mostly plasma cells admixed with polymorphonuclear cells (H&E stain, magnification x400). At the time of death, no evidence of rejection or GVHD was present.

Upper panel. Histopathologic specimen from an animal that died on day 132 posttransplant (simultaneous protocol with drugs). The blood cell counts demonstrated anemia and an increase of WBCs. Histology in the lung revealed focal interstitial infiltration by dense sheets of lymphoid cells (H&E stain, magnification x100). High power demonstrates large variation in nuclear size, hyperchromasia, and irregularity of nuclear rim, which are characteristics of lymphoma (H&E stain, magnification x400). The lymphoma was also present in the spleen and lymph node. At the time of death, no evidence of rejection or GVHD was present.

Evaluation of donor-specific tolerance in vitro and in vivo in chimeras receiving limb transplants:

MLR assays were performed in all animals, including three animals in group 4. Evidence of donor-specific tolerance in vitro was established by the one-way MLR assay. In all three groups (3, 4, and 5), splenocytes from (ACI→WF) chimeras were hyporesponsive toward donor (ACI) alloantigens with brisk reactivity to third-party (Fisher) rat splenocytes ($P < 0.05$). The proliferation responses (expressed as CPM + SEM) are summarized in Figure 4. Tolerance and immunocompe-

tence were confirmed in vivo by prolonged survival of donor-specific ACI limb transplants. The Kaplan-Meier life table analysis of the groups is shown in Figure 5.

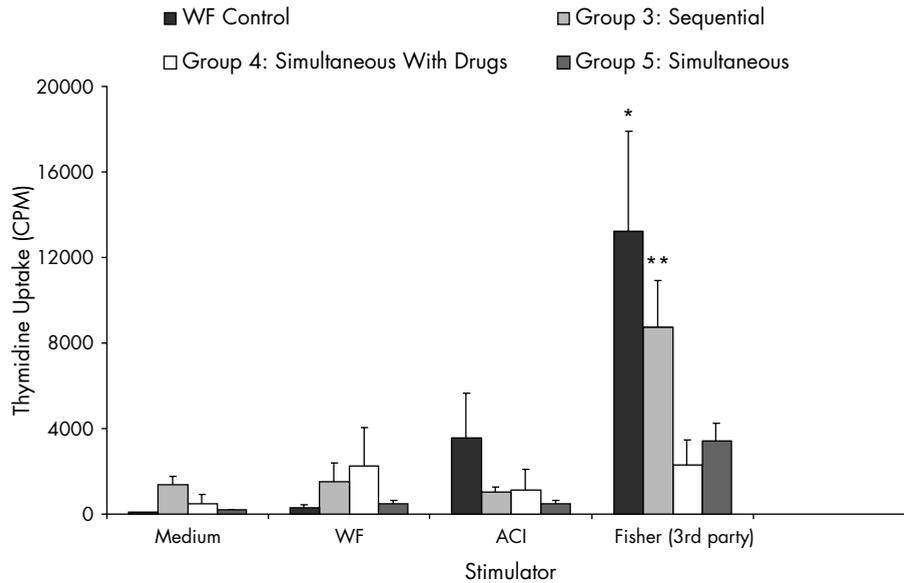


Figure 4. Mixed lymphocyte reactivity assay for in vitro tolerance. Naïve WF controls (black bars), group 3 (light gray bars), group 4 (open bars), and group 5 (dark-gray bars). Compare results of anti-donor reactivity in groups 3 (light gray bars) and 5 (dark-gray bars); chimeras in both groups that received irradiated limbs demonstrated excellent reactivity toward third-party (Fisher rat) antigens with donor-specific (ACI) hyporesponsiveness. In contrast, depressed reactivity toward both donorspecific (ACI) and third-party (Fisher rat) antigens is seen in group 4 (open bars). Results are expressed as counts per minute (CPM) + standard error of mean (SEM). *Significant difference in proliferation comparing reactivity toward Fisher cells, with reactivity toward ACI ($P \leq 0.01$) and WF cells in controls ($P \leq 0.05$). **Significant difference in proliferation comparing reactivity toward Fisher cells, with reactivity toward ACI cells controls ($P \leq 0.01$) in group 3 controls.

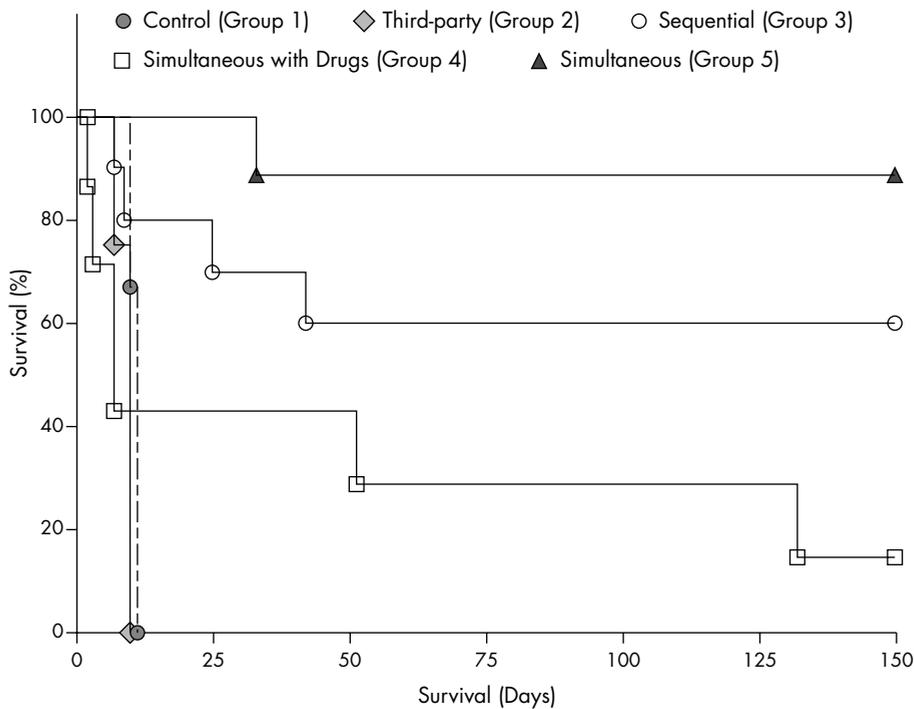


Figure 5. Survival of graft transplants (Kaplan-Meier life table method). Naïve WF rats that received ACI limb without treatment (group 1, grey circle) rejected their limbs in 5.7 ± 1.5 days. Chimeras transplanted with third-party limbs (group 2, rhombus) rejected their limbs in 7.3 ± 1.5 days. Chimeras transplanted with donor ACI limbs (group 3, open circle) demonstrated prolonged survival of limbs (158 ± 2.3 days) until the end point of the study. Results in group 4 (open square) indicate that 70% of animals died before 100 days after limb transplantation. Graft survival was determined by daily visual examination for signs and symptoms of rejection. Mean survival of group 4 (open square) was 50.7 ± 24.6 days whereas that of group 5 (triangle) was 160.5 ± 3.6 days. Percentage survival of donor-specific grafts was 100% at the end point of the study. Rejection of Fisher rat grafts (rhombus) was brisk and comparable to controls.

DISCUSSION

Previous studies have shown permanent acceptance of donor-specific pancreatic islets transplanted simultaneously with donor BM across allogeneic⁵ or xenogeneic barriers¹⁹ in lethally conditioned rodents. Similar results were also achieved after nonlethal conditioning in an MHC-mismatched mouse model²⁰. Pancreatic islet allografts transplanted within 24 hr of donor BMT survived for more than 200 days without evidence of chronic re-

jection or recurrent insulinitis. The transplanted islets were functional and maintained glucose homeostasis. Host mice rejected MHC disparate third-party islet allografts, demonstrating donor specificity of tolerance. Other experimental studies have demonstrated that donor BM infusions performed simultaneously with organ transplantation may have a “protective” effect and can augment chimerism or donor-specific hyporeactivity across allogeneic³ and xenogeneic²² barriers. Corroborating such experimental evidence, several clinical studies have reported similar results in kidney²³, heart and lung²⁴, and pancreas²⁵ transplants.

Similar reports in the literature are scarce in the field of composite tissue allotransplantation. A few studies have shown that donor-specific skin grafts placed at the time of allogeneic BM reconstitution are permanently accepted without rejection. In one study, full-thickness skin grafts were transplanted simultaneously with BM in ablated mice (B10BR→B10). Donor-specific skin grafts were permanently accepted without rejection. Histopathologic examination of skin grafts 90 days after transplantation showed mild infiltration of mononuclear cells and neutrophils without clinical evidence of acute or chronic rejection. In vitro assays showed donor specificity of tolerance and full reactivity to third-party cells²⁶. In another study, skin transplantation was performed within 1 hr of chimerism induction in fully mismatched mice. All chimeric mice permanently accepted host and donor type skin grafts and promptly rejected third-party skin grafts²⁷. On the basis of such reports and on our own experience in manipulating the individual elements (techniques and sequence) in the conditioning protocol, our present experiments demonstrated that an effective simultaneous protocol could theoretically be used in the clinical setting.

We previously demonstrated that mature immunocompetent T cells from the BM or lymph nodes in the donor CTA could cause lethal GVHD in established, stable chimeras after limb transplantation²⁸. Irradiation of the donor limb before transplantation eliminated GVHD in tolerant chimeras. Our experiments in group 3 confirmed that such a conventional protocol of MAC induction and irradiated CTA transplantation could lead to prolonged survival. In group 4, we used immunosuppression with tacrolimus and MMF in addition to radiation during conditioning, with the rationale that these drugs would prevent acute rejection of both the CTA and donor BM while the chimeric state was being established. In group 4, we established high levels of donor chimerism. However, 70% of animals died before day 60. We interpreted our results on the basis of evidence obtained (highly depleted RBC, WBC, and platelet counts), the probable cause of these deaths was the lack of engraftment of the BMC infusion and therefore the lack of

proper reconstitution of the animals. However, it has been shown that MMF and irradiation can cause aplastic anemia in rodents²⁹. The combination of immunosuppression with 950 cGy of irradiation could have resulted in aplasia of BM and early death of some hosts. The donor HSCs from the infused BM "rescued" some hosts from the initial effects of conditioning and resulted in stable chimerism and prolonged survival. However, this experiment did not establish the exact mechanisms as to why some animals succumbed to aplasia and others did not. Even though engraftment of HSC is important for establishment of MAC, it is the stability of this mixed chimeric state that is responsible for the tolerant state.

To confirm whether immunosuppression coupled with irradiation was the cause for early mortality of most of the animals in this group, we repeated the same experiment but without the drugs in group 5. Here we determined whether 950 cGy of conditioning allowed reliable engraftment of fully allogeneic BM without the additional need for drugs. We demonstrated 100% engraftment with high levels of stable MAC, no evidence of GVHD, and survival for more than 150 days. In the present study, all the limbs used in the experimental groups were lethally irradiated. However, others and our group have proposed that the BM contained in a transplanted limb can serve as a donor BM source to induce stable levels of MAC without BM cell infusion. In other experiments, we have taken advantage of the BM contained in vascularized femurs and induced high levels of chimerism in partially conditioned recipients (Laurentin-Perez et al., unpublished data, 2002).

In addition to being tolerant, 9 of 10 animals in group 5 showed prolonged survival. Moreover, the fact that survival was excellent despite the fact that the animals were not housed in a barrier facility indicates a state of relatively robust immunocompetence. To confirm in vitro reactivity to donor and third-party antigens, we performed MLR assays and demonstrated donor-specific hyporeactivity and vigorous third-party reactivity, both prerequisites for robust tolerance. We found that MLR reactivity in the simultaneous group (group 5) was similar to that demonstrated by sequential chimeras (group 3). Also, we found that the mixed chimeras demonstrated robust immunocompetence in vivo to third-party grafts and prolonged acceptance of donor limbs. On clinical examination of group 5, the lack of clinical evidence of acute rejection or acute GVHD was corroborated by histopathology evidence. This study therefore demonstrates that our long-term survivors of a simultaneous protocol were free from both acute rejection and GVHD.

CONCLUSION

Our results demonstrate that hind-limb transplantation simultaneous with BMT leads to development of high levels of stable mixed chimerism and induces robust tolerance to a transplanted CTA across a fully mismatched antigenic barrier. Experimentally and clinically, MAC-induced tolerance has been widely tested in solid-organ transplants³⁰. With the success of our study, the applicability of MAC-based tolerance protocols in the field of composite tissue allotransplantation has moved one step closer to the clinical scenario.

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Chapter 8

Lymphadenectomy prior to rat hind limb allotransplantation prevents graft-versus- host disease in chimeric hosts

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INTRODUCTION

Composite tissue allograft (CTA) procedures would provide optimal treatment for patients suffering from large tissue defects caused by trauma, tumor resection or congenital defects by providing identical tissue parts for reconstruction. In CTA procedures, multiple tissue types such as skin, subcutaneous tissue, nerve, blood vessels, lymphatics, bone and muscle are transplanted in the form of extremities, larynx or facial tissues. The major disadvantage of these procedures is the need for immunosuppressive drugs to prevent rejection. These toxic drugs are associated with an increased occurrence of neoplasms, opportunistic infections, end-organ toxicity and do not prevent chronic graft rejection effectively¹. Although these risks are considered to be justified in life-saving organ transplant procedures, their use in life-enhancing reconstructive CTA procedures remains controversial².

A promising approach to successfully eliminate the need for toxic immunosuppressive drugs is through induction of donor specific tolerance, which possibly could make composite allotransplantation the preferred treatment for many reconstructive procedures. One of the best-studied and most effective methods of inducing tolerance is through mixed allogeneic chimerism (MAC) achieved by bone marrow (BM) transplantation³. In spite of the promising potential of MAC, there still exist complications associated with its use that must be overcome to achieve widespread clinical application. One such complication is that chimeric hosts develop graft-versus-host disease (GVHD) after transplantation of hind limb allografts. Experimentally, non-chimeric and chimeric transplant models have been used to study GVHD. The F₁ hybrid model is the classic non-chimeric transplant model used, in which hosts develop GVHD after transplantation of lymphocyte-rich allografts⁴⁻⁷. Similarly, chimeric hosts have also been shown to be susceptible to GVHD after transplantation with these types of allografts⁸⁻¹¹.

Tolerance induction through MAC cannot be applied clinically in CTA procedures unless this complication associated with its use is overcome. Risk factors associated with GVHD are the amount of transplanted lymphocytes, histocompatibility, host and donor age, and host environmental factors such as bacterial and viral contamination. Protocols aimed at preventing GVHD in experimental animals and in the clinical setting have focused mainly on reducing the number of donor lymphocytes within the graft. In BM transplantation, GVHD has been effectively prevented by ex-vivo T-cell depletion (TCD) and UV-irradiation^{12,13}. In small bowel transplantation, GVHD has been prevented by irradiation of grafts^{5,14} lymphadenectomy⁴ or donor pretreatment with antilymphocyte serum or anti-T-cell monoclonal antibodies¹⁵. Foster et al.⁸ speculated that intact BM in CTAs was responsible for

GVHD in chimeric hosts, whereas Hewitt et al.¹⁶ speculated that BM or other tissues, such as lymph nodes (LNs), were responsible for GVHD in F₁ hybrid hosts.

The purpose of this study was to determine in mixed chimeric hosts whether the BM and/or LNs within transplanted limbs carry a large enough lymphocyte load to cause GVHD. We tested the feasibility of surgically excising the LNs to eliminate the threat of GVHD in chimeric hosts receiving CTAs. This approach could be a better alternative to graft irradiation for prevential GVHD.

MATERIALS AND METHODS

A total of four groups were used. Three groups of [ACI→WF] chimeric rats received ACI hind limbs that were irradiated (1050 cGy), non-irradiated, or had all LNs removed. All animals were inspected daily for signs of rejection and GHVD. Group 4 (naïve ACI rats) was used to enumerate lymphocytes present in BM and LNs of hind limb.

Animals: Male (5-7 week) ACI (RT1 A^b) and Wistar Furth (WF, RT1 A^u) rats weighing between 200-350 g were used. Animals were housed in separate cages at 24°C, with light 12 hrs a day and in air-flow regulated rooms. They were fed standard rat chow and given water ad libitum. All handling of animals was done in accordance with the guidelines of the Animal Care and Use Committee of the Louisville School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86-23).

Groups: In Groups 1, 2, 3, [ACI→WF] chimeras were prepared by irradiating host WF rats with 950 cGy of total body irradiation (TBI) and reconstitution with ACI BM (depleted of $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells). Donor limb transplantation was done at least 28 days after BM reconstitution. In Group 1 (controls, n=10), host [ACI→WF] chimeras were transplanted with non-irradiated limbs from donor ACI rats that were syngeneic to the BM donor. In Group 2 (controls, n=8), host [ACI→WF] chimeras were transplanted with irradiated (1050 cGy) limbs from donor ACI rats that were syngeneic to the BM donor. In Group 3 (n=6), host [ACI→WF] chimeras received non-irradiated ACI limbs from which the popliteal and inguinal LNs were surgically removed prior to transplantation. In Group 4 (n=3), six ACI limbs

were harvested to calculate and characterize the cells present in the BM and LNs of one hind limb.

T-cell depletion (TCD) of BM in vitro: TCD was carried out as described elsewhere⁹. Briefly, aliquots of 200×10^6 unseparated ACI BM cells were incubated with purified anti- $\alpha\beta$ -TCR monoclonal antibodies (MoAb) (R73; mouse IgG₁; BD PharMingen) and anti- $\gamma\delta$ -TCR MoAb (V65; mouse IgG₁; BD PharMingen) for 30 min at 4°C. Cells were incubated for 60 min at 4°C with immunomagnetic beads (Dynabeads M450, Dynal ASA, Oslo, Norway) at a bead/T-cell ratio of 20:1 and placed in a magnetic cell separator for 2 min to negatively select T cells. Cells were washed, counted and resuspended in Medium 199 (Life Technologies, Rockville, Md., USA) plus gentamycin at a concentration of 100×10^6 BM cells per ml.

Verification of bead depletion using flow cytometry: To confirm the adequacy of TCD, aliquots of BM cells were set aside for analysis prior to TCD, after incubation with primary MoAbs, and after TCD. Cells were incubated with either anti- $\alpha\beta$ -TCR FITC (R73; mouse IgG₁; BD PharMingen), anti- $\gamma\delta$ -TCR FITC (V65; mouse IgG₁; BD PharMingen) or rat-adsorbed goat anti-mouse IgG FITC (BD PharMingen) for 30 min and analyzed on a fluorescence activated cell sorter (FACS Calibur, Becton Dickinson, Belford, Mass., USA).

Preparation of mixed allogeneic chimeras [ACI→WF]: Mixed allogeneic chimeras were prepared according previously established methods¹⁶. Briefly, WF hosts were conditioned with unfractionated 950 cGy of TBI. Using sterile technique, irradiated hosts were reconstituted within 4 to 6 hours of TBI, with 100×10^6 ACI rat BM cells (diluted in 1 ml modified Eagle's medium) via penile vein infusion.

Characterization of chimerism after BM reconstitution: Engraftment of allogeneic BM was confirmed 4 weeks after BM reconstitution using flow cytometry to determine the percentage of peripheral blood lymphocytes (PBL) bearing ACI or WF major histocompatibility complex (MHC) Class I antigens. Whole blood aliquots of 100 μ l were stained with anti-RT1A^{abl} FITC (B5 LOU/cN IgM, BD PharMingen) and purified anti-RT1A^u (NR3/31, rat IgG_{2a}, Serotec) MoAb for 30 min. Cells were washed and fixed in 1% paraformaldehyde. The threshold for detection of donor cells was 0.5%. Flow typing was repeated at 60 and 90 days after BM reconstitution to confirm stable chimerism before limb transplantation.

Irradiation of donor limbs: ACI donors were treated with 1050 cGy of TBI. Hind limbs were procured from these animals after the TBI and served as donor limbs.

Hind limb transplantation: Donor (ACI) animals and host [ACI→WF] chimeras were anesthetized with pentobarbital 60 mg/kg.

Donor operation: The skin was incised proximally to the mid-thigh area, the femoral artery, vein and nerve were dissected, and the individual muscle groups divided proximally. The limb was flushed for 10 min with heparinized Ringer's Lactate. The femur was divided at the mid-shaft.

Host operation: The hind limb was removed in a similar fashion as described above and the donor femur was fixed using a 2 mm Kirschner wire. Femoral vessels and nerves were anastomosed using microsurgical technique (10-0 Nylon). The muscles and tendons were approximated using 5-0 Nylon, and the skin was closed using absorbable suture (5-0 Monocryl). Automutilation was prevented by daily spraying a Chew Guard solution (Summit Hill Laboratories, Navesink, N.J., USA) on the transplanted, insensate limb for 60 days.

Surgical removal of lymph nodes: Studies were carried out to localize all LNs within the rat hind limb. Lymphatics were selectively stained by injecting 0.5-1.0 ml isosulfan blue 1% dye (Ben Venue Labs, Bedford, Ohio, USA) into the footpad, using a 30 Gauge needle. Shortly thereafter, the limb was dissected and the LNs identified. With the information from these studies, we could confidently remove all LNs from the hind limb prior to transplantation using microsurgical techniques.

Characterization of chimerism after limb transplantation: Chimeras were characterized by flow cytometry of PBL after limb transplantation at 15, 30, 60, 120 and 150 days to determine levels of donor macrochimerism.

Clinical and histopathologic assessment for rejection and GVHD: Animals were monitored daily for signs of acute rejection of the limb and for signs or symptoms of acute or chronic GVHD. Important clinical signs of rejection included edema, erythema, escharification and necrosis¹⁷, and signs of GVHD included dermatomyeloma, weight loss, diarrhea, or general unkempt appearance⁹. Histopathologic grading for

cutaneous rejection and GVHD were based on previously described criteria⁹. Skin and muscle biopsies from the limb CTA were taken at 14 days, 28 days and every month thereafter, and ear wedge skin biopsies were taken once every month. All animals were weighed daily and assessed visually for signs of rejection and GVHD. Target tissues for GVHD including tongue, ear, liver and small intestine were harvested at the end of the study, fixed in 10% buffered formalin and processed routinely for hematoxylin and eosin (H&E) staining. As previously reported, allogeneic CTA hosts manifested the most severe characteristics of GVHD in skin and tongue specimens⁷. Accordingly, these tissues were evaluated for GVHD in our experiments.

In vitro assessment of tolerance: Mixed lymphocyte reaction (MLR) assays were done at the end of the study and/or when the animals were killed. Spleens were sterile harvested, crushed, and the isolated lymphocytes were ACK-lysed, washed and resuspended in cMLR medium. Cultures were incubated at 37°C in 5% CO₂ pulsed on the fourth day with 1 μCi [³H] thymidine (PerkinElmer, Boston, Mass., USA), harvested on the 5th day with an automated harvester (PHD Cell Harvester, Technology, Cambridge, Mass., USA) and counted in a beta scintillation counter (Beckman, Palo Alto, Calif., USA). Results were expressed as counts per minute (CPM) + SEM.

In vivo assessment of tolerance: Three to six months after BM reconstitution, [ACI→WF] chimeras were transplanted with non-irradiated ACI limbs to assess for donor specificity of tolerance. Skin grafting was performed on non-transplanted chimeras and on chimeras of group 3 (n=2) to test donor specificity of tolerance. Full-thickness skin grafts (1 cm diameter) were harvested from the dorsum of ACI and (third-party) Fisher donors and placed on each recipient animal, separated by a 3-mm skin bridge. Tapes were carefully removed and grafts were scored daily for rejection.

Enumeration of cells within BM and LNs: BM was flushed from femoral and tibial bones taken from ACI rats with DMEM (Life Technologies, Rockville, MD, USA) and the inguinal and popliteal LNs were dissected and crushed between frosted slides in DMEM. Cells were counted and the total amount of BM and LN cells per limb was calculated. Samples of BM and LN cells were taken to enumerate the percentage of αβ TCR⁺ T cells and B cells. Aliquots of 1 × 10⁶ were stained with anti-αβ-TCR PerCP MoAb

(R73, mouse IgG₁; BD PharMingen) and anti-CD45RA PE MoAb (OX-33, mouse IgG₁; BD PharMingen) for 30 min and analyzed using FACS.

Killing criteria: Animals were killed at 150 days, which was the end point of the study. Animals with obvious signs of rejection or GVHD and/or failure to thrive were killed when these signs appeared.

Statistical analysis: Continuous variables were expressed as means \pm SEM, and experimental data was evaluated for significant differences using analysis of variance (ANOVA) and the post-hoc Tukey's test. Differences were considered to be significant if $P < 0.05$.

RESULTS

FACS analysis was performed before transplantation to detect the percentage of PBL bearing ACI or WF MHC Class I antigens. All irradiated WF hosts reconstituted with TCD donor ACI rat BM cells demonstrated high levels of chimerism ($85 \pm 3\%$). Chimerism levels remained stable in the animals that received limbs from which all LNs were excised, (group 3) which was similar to the non-GVHD control animals that received irradiated limbs (group 2). In contrast, GVHD-control animals (group 1) showed more than 15% increase in chimerism.

Clinical detection of GVHD after limb transplantation in chimeric hosts: As in previous studies^{9,10}, weight loss was the most reliable predictor for the onset and progress of acute GVHD. In control group 1, chimeras transplanted with non-irradiated hind limbs succumbed to GVHD at 22.4 ± 0.8 days post-transplantation. These animals lost over 25% of their initial weight during the first 3 weeks and had to be killed. In control group 2, none of the animals receiving irradiated (1050 cGy) hind limbs developed clinical or histopathologic signs of acute or chronic GVHD. These animals also experienced some weight loss (approximately 10%) during the first 2 weeks following limb transplantation, but they gradually regained weight and recovered, thriving for over 150 days post-transplantation. In group 3, chimeras transplanted with lymphadenectomized limbs, showed a similar weight loss and gain pattern as group 2. None of these animals developed signs of acute GVHD, and all survived over 150 days post-transplantation. However, in 2/6 animals, mild clinical signs of a possible graft-versus-host (GVH) response were observed. These signs were

less apparent hair growth, a slight scruffy appearance, and mild weight loss (approximately 15%) compared to group 2, but without diarrhea, dermatoerythema, or hyperkeratosis. Clinical appearance of rats in group 3 is compared to that of group 1 (GVHD controls) in Fig. 1.



Figure 1. Clinical assessment of GVHD.

Upper. Lethal GVHD in chimeras receiving non-irradiated limbs (group 1). Note the severe dermatoerythema on the abdomen and non-transplanted paw and its scruffy appearance at 21 days post-transplantation.

Right page. No signs of GVHD and prolonged survival in chimeras receiving lymphadenectomized limbs (group 3).



Histological detection of GVHD after limb transplantation in chimeric hosts: Histopathological examination of skin and tongue from animals that received limbs without lymph nodes did not reveal signs of GVHD. In Fig. 2, representative histopathological findings are compared to those of the controls (group 1 and 2) and animals from group 3.

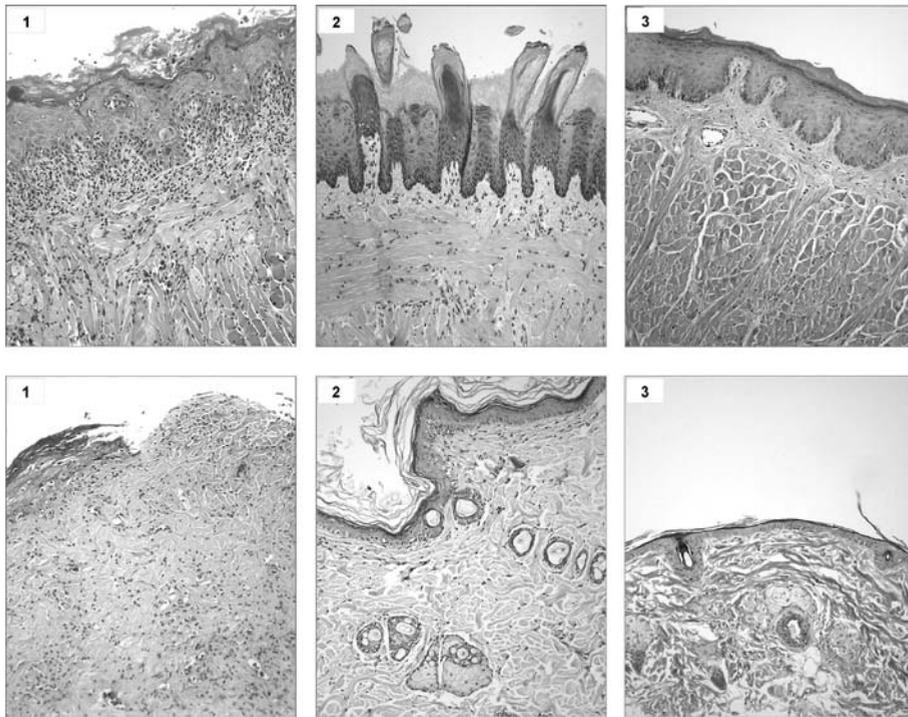


Figure 2. Histopathologic assessment of GVHD. H&E sections of tongue and ear/skin of groups 1, 2, 3 are compared (x400).

Upper panel. Tongue. In group 1 moderate to marked mononuclear cellular infiltration is noted in the epithelium and, particularly, the lamina propria. Chronic inflammatory infiltrates were also noted within the myocytes (myositis) with myocyte necrosis consistent with GVHD. Dyskeratosis and vacuolated epithelial cells were also noted. Similar to the skin sections, specimens from the remaining groups (2 and 3) demonstrated normal epithelium, lamina propria, and myofibrils.

Lower panel. Skin. In group 1, specimens revealed scattered slight-to-moderate mononuclear cellular infiltrates within the dermis, necrosis and ulceration of the epidermis, dermal fibrosis, and loss of adnexa. Specimens shown from the remaining groups (2 and 3) demonstrate normal epidermis, dermis, and adnexal structures and lack of effects related to GVHD.

Assessment of donor-specific tolerance in vivo and in vitro after limb transplantation: No clinical signs of rejection were observed in ACI limbs transplanted to [ACI→WF] chimeras, 3-6 months following BM reconstitution (groups 2 and 3). Histologic examination of skin (from CTA) and muscle biopsies performed at regular intervals during the experimental study confirmed the lack of rejection, which is further evidence of the tolerant state of these long surviving chimeras. In addition, prolonged

survival of donor specific skin grafts in [ACI→WF] hosts (group 3) and vigorous rejection of third-party skin grafts from fully mismatched Fisher rats confirmed tolerance and immunocompetence in vivo (Fig. 3).

Evidence of donor specific tolerance in vitro was established by the one-way MLR assay. Splenocytes from [ACI→WF] chimeras that received limbs without lymph nodes (group 3) showed hyporesponsiveness toward donor (ACI) with intact and significant reactivity toward third-party Fisher rat splenocytes ($P < 0.05$). The proliferation responses (expressed as CPM) from the MLR assays performed in groups 2 and 3 are summarized in Fig. 3.

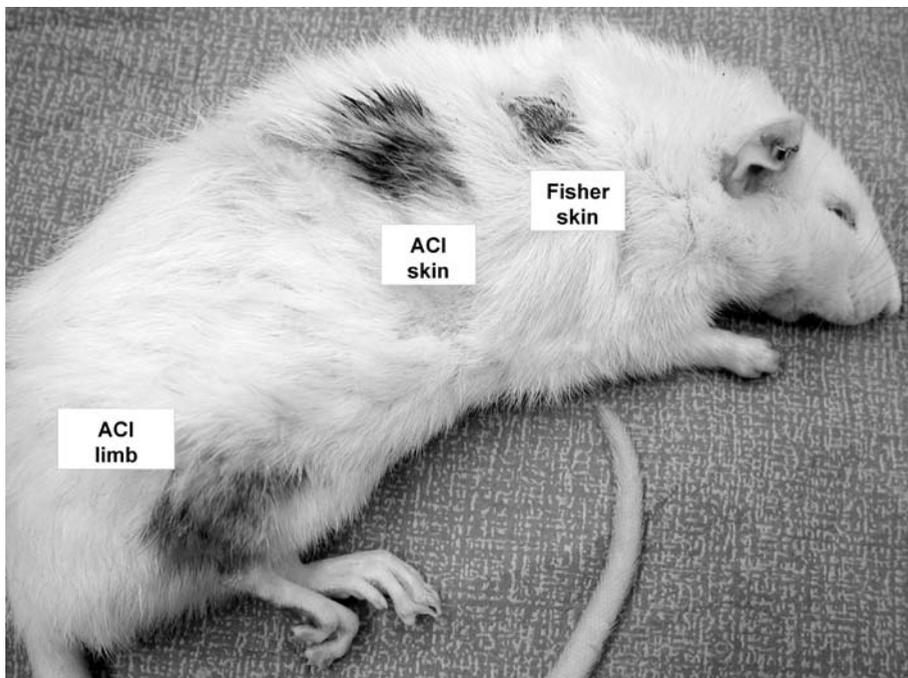
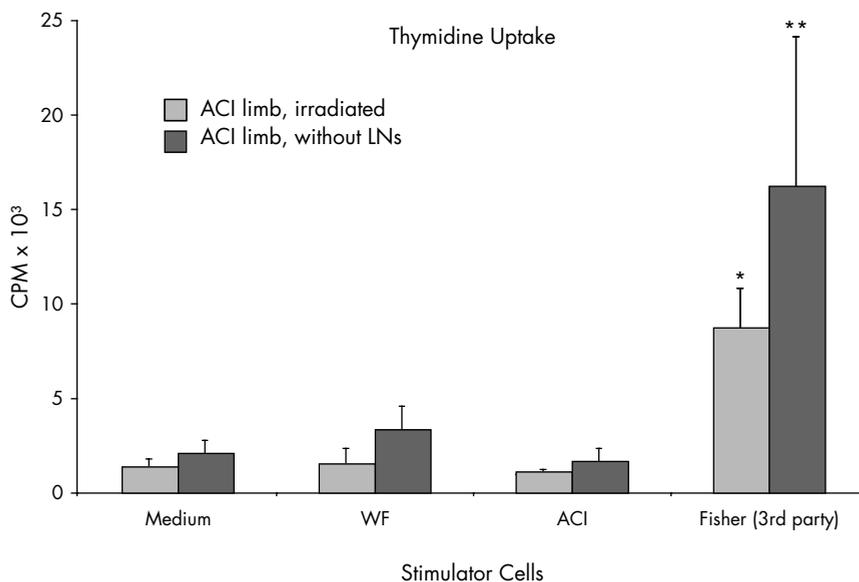


Figure 3. Immunocompetence test of hosts.

Above. Photo shows a [ACI→WF] chimera that received a non-irradiated lymphadenectomized ACI limb (group 3). To test for donor-specific tolerance and third-party reactivity in vivo skin grafting was performed 150 days after limb transplantation in two animals. Fisher skin grafts were promptly rejected, while ACI skin grafts were accepted. Note the necrotic Fisher skin graft and the abundant hair growth of the ACI skin graft.



Above. Graph shows results of mixed lymphocyte reaction (MLR) assay from groups 2 and 3, quantified in counts per minute (CPM) + SEM. Chimeras that received irradiated or lymphadenectomized limbs demonstrated excellent reactivity towards third party antigens with donor specific (ACI) hyporesponsiveness. Baseline counts in cMLR medium are shown for comparison. GVHD in chimeras transplanted with non-irradiated limbs led to persistent and severe immuno-incompetence (not shown). * Indicates significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward ACI ($P < 0.05$) in group 2. ** Indicates significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward ACI cells ($P < 0.05$) in group 3. In both groups no difference was present in reactivity toward ACI cells when compared to reactivity toward WF cells, indicating donor specific tolerance towards ACI.

Location of LNs and enumeration of T and B cells in BM and LNs: Consistently, a single popliteal LN was found in each limb by dye injection. In our model, the inguinal fat pad flap is also transplanted to cover the anastomosed vessels. This fat contained three to four small inguinal LNs. With this information, all LNs could be easily removed prior to transplantation using microsurgical techniques (Fig. 4). The popliteal plus inguinal LNs of a single limb contained $117 \pm 16 \times 10^6$ cells and the BM from femoral and tibial bones $194 \pm 25 \times 10^6$ cells (group 4). The percentage of $\alpha\beta$ -TCR⁺ T cells and B cells within the LNs was $61.3 \pm 4.4\%$ and $20.6 \pm 3.6\%$ and in the BM $5.0 \pm 0.6\%$ and $31.2 \pm 2.2\%$ respectively.

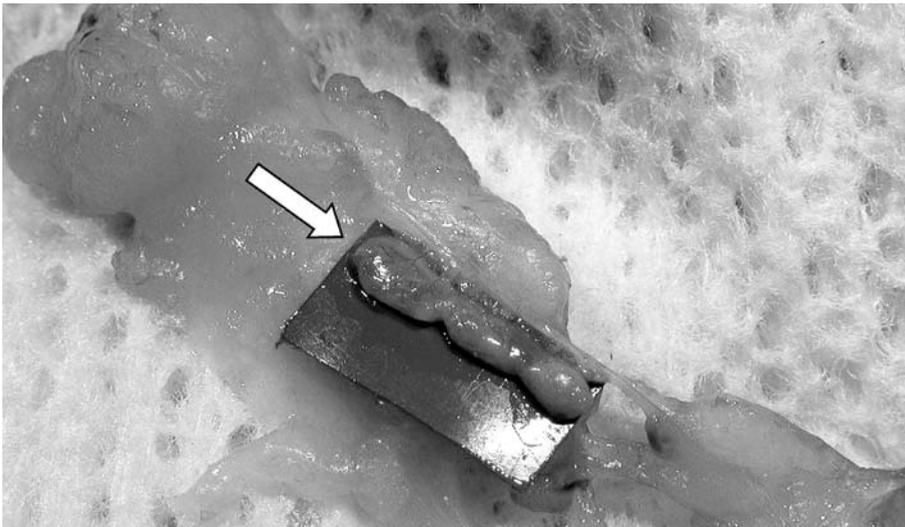
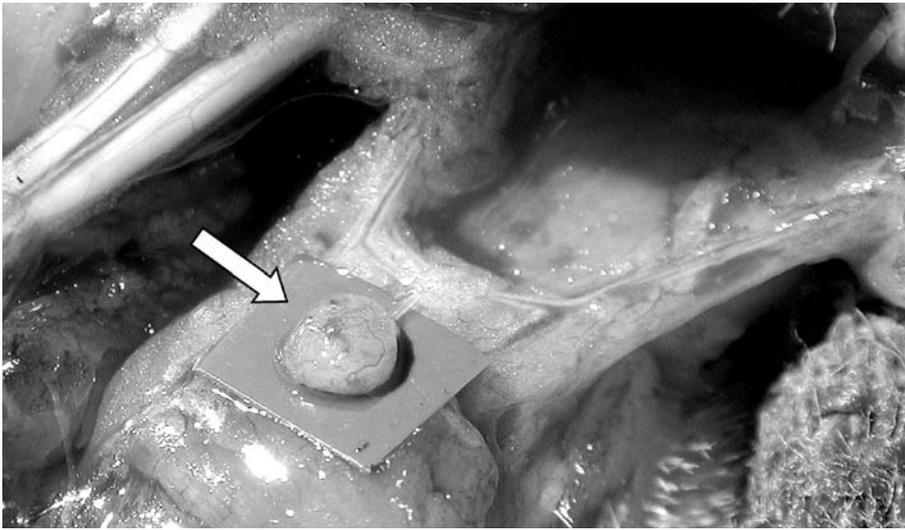


Figure 4. Excision of lymph nodes prior to limb transplantation.

Upper. Single dissected LN in the popliteal space.

Lower. Three dissected LNs in the inguinal fat pad are shown prior to excision (*arrow*). The vascular supply of the LNs and lymphatic vessels were carefully transected and the lymph nodes removed.

DISCUSSION

GVHD is a common phenomenon in allogeneic BM transplantation and represents a major cause of post BM transplant morbidity and mortality. Hosts of organ transplants are also at risk for GVHD, however, they are relatively immunocompetent, and the grafts usually do not contain large numbers of lymphocytes. As a result, the host-versus-graft (HVG) reaction is far stronger than the GVH component of this bi-directional reaction. Therefore, GVHD occurs only sporadically after solid organ transplantation, but has been reported following liver, kidney, heart-lung and multivisceral organ transplantation¹⁸. Transplantation of a lymphocyte rich organ such as the small intestine causes GVHD more frequently. The mortality rate, once GVHD occurs, is around 40% for solid organ transplantation recipients¹⁸.

Introduction of composite tissue allotransplantation into the clinical arena could also come with the risk of GVHD due to the lymphocyte content in these grafts. In this new type of reconstructive procedure, multiple tissues are transplanted to reconstruct severe injuries and deformities of extremities, larynx or head and neck region. Even a small risk of GVHD in these reconstructive procedures would hamper its widespread clinical applicability. Current methods used clinically to prevent rejection of CTAs rely on toxic immunosuppressive drugs, which are a necessity to achieve prolongation of allograft survival. The ultimate goal is to replace these toxic immunosuppressive drugs with transplantation tolerance. However, if we do so, immunosuppression of the GVH reaction is also removed, and GVHD may occur and present a greater problem. Though GVHD does not occur in chimeras undergoing transplants such as skin¹⁹, heart²⁰, lung²¹, and kidney²², it is well documented following transplantation of lymphocyte-rich grafts such as small intestine¹¹ and certain CTAs⁸⁻¹⁰. In previous studies we found that 10/10 chimeric hosts died of GVHD following hind limb transplantation⁹. In a similar study reported by Foster et al. 1/9 animals developed lethal GVHD⁸. Hewitt et al. found that 37.5% of rat hind limb hosts developed lethal GVHD in a reverse, one-way parental to F₁ hybrid model¹⁶. In contrast, most studies using modern long term immunosuppressive regimens report the absence of (lethal) GVHD following rat hind limb transplantation non-chimeric hosts^{17,23,24}. One study documented only transient GVHD in 33.5% of rat hind limb hosts maintained on FK506²⁵, others noted non-lethal chronic GVHD in 30% of hosts receiving short term course of FK506²⁶. Lethal GVHD after limb transplantation was reported in one study with long term CsA therapy²⁷. From this data it is clear that substitution of immunosuppressive drugs by donor specific tolerance may increase the risk of

GVHD when lymphocyte-rich grafts are transplanted. Tolerant hosts allow donor T cells transferred with the graft to attack the host unhindered, making these animals particularly susceptible to GVHD. Several approaches exist to eliminate the GVH response in tolerant hosts, and they are generally based on eliminating or reducing the number of lymphocytes transplanted with the graft. Methods used to avoid GVHD following small intestine transplantation include irradiation of the graft⁵ or mesenteric LNs¹⁴, removal of mesenteric LNs⁴ and donor treatment with antilymphocyte serum or anti-T cell monoclonal antibodies¹⁵. In our rat CTA model, we have successfully eliminated GVHD by radiating (1050 cGy) the hind limb prior to transplantation to the chimeric host¹⁰. We confirmed that the cells causing GVHD are radiosensitive and therefore eliminated by this procedure.

The tissues transplanted with the rat hind limb represent most tissues that would be included in many clinical CTA procedures. It has been speculated that of these tissues, BM⁸ or the LNs¹⁶ are responsible for GVHD seen in tolerant hosts. In the present study we developed a unique and clinically applicable alternative for graft radiation that could be used in certain types of CTAs. We investigated whether we could reduce the number of T cells transplanted with the limb and avoid GVHD using a selective surgical technique.

In the experiments described here we have demonstrated that removal of all LNs prior to transplantation decreases the amount of T cells transplanted with the limb dramatically, as is evidenced by the absence of GVHD in chimeric hosts. In addition, this indicates that the T cell content of the BM and other tissues of these lymphadenectomized limbs, such as skin, muscle and vascular bed, was not sufficient to cause GVHD, contradicting previous hypotheses. Two chimeric hosts that received limbs without LNs showed post-operative weight loss of approximately 15% during follow-up, combined with less abundant hair growth and a slight scruffy appearance. Though GVHD was not confirmed histologically, a mild GVH reaction could have been responsible for these signs. Since the severity of GVHD is closely related to the logarithm of the number of T cells in the graft²⁸, it is possible that more T cells remained in these transplanted hind limbs resulting in a mild GVH response.

To determine conclusively that in our limb model, the majority of mature T cells responsible for GVHD reside in the LNs and not the BM, we counted and enumerated the phenotype of cells from these respective tissues. The BM is mainly present in the femur and tibia, and typically one popliteal LN and 3-4 inguinal LNs are found. In rats, the majority of mature T cells are $\alpha\beta$ -TCR⁺ and we showed that the percentage of $\alpha\beta$ -TCR⁺ T cells in LNs is

approximately 12-fold of that in BM, whereas the total cellularity of BM is only 1.5-2 times greater than that of the LNs in one limb. These findings for the first time demonstrate that GVHD in tolerant hosts transplanted with hind limb allografts is caused by the large quantity of lymphocytes within the LNs that come with the graft. However, in this study we did not rule out the possibility that the microenvironment within the lymph nodes plays a role in the development of GVHD from BM derived lymphocytes. Future studies will need to explore the interaction between the lymph nodes microenvironment and lymphocytes in the pathogenesis of GVHD.

This study, like others, shows that induction of donor specific tolerance using BM chimerism can eliminate the need for toxic immunosuppressive drugs and prevent acute and chronic allograft rejection, while retaining third-party immunocompetence. All long-surviving chimeric hosts in our study, demonstrated donor specific tolerance and immunocompetence in both in vitro assays and in vivo assessment. In addition, histopathologic examination of tissue for signs of rejection or GVHD confirmed that these chimeras were tolerant and without GVHD. Donor specific skin grafts placed 150 days after limb transplantation were accepted indefinitely, while third party skin grafts were promptly rejected.

Prevention of GVHD in chimeric hosts using lymphadenectomized limbs turned out to be a straightforward procedure that provided a simple alternative to toxic graft irradiation which potentially could lead to tumor formation in CTAs. No long-term deleterious effect on lymphatic drainage of limbs were observed following lymphadenectomy. Although we did see edema early after transplantation, this edema resolved after 2 weeks. These observations were consistent with hind limb transplantation studies in which edema and lymphatic regeneration has been reported in non-lymphadenectomized limbs²⁹. The present findings indicate that CTAs containing LNs could potentially induce GVHD in experimentally or, in future, clinically tolerized hosts. Though the percentage of mature T cells in human BM is higher than in rats, these findings demonstrate that CTAs without any LNs, such as a hand, almost certainly would not result in GVHD, especially when maintained on immunosuppressive drugs. This is also confirmed by clinical hand transplant recipients and indicates that irradiation of the donor hand, as performed by one clinical hand transplant team, was not necessary³⁰. Furthermore, localization and removal of LNs from the graft could be aided by dye injection, which is clinically used to identify the sentinel LN of certain tumors. This technique could prove to be beneficial for the prospective transplantation of entire extremities and/or head and neck CTAs to individuals made tolerant for these grafts.

ACKNOWLEDGEMENTS

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Chapter 9

A model for free microvascular lymph node transplantation in rats

SUBMITTED

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INTRODUCTION

The advance in microsurgical technique has made it possible to transplant tissues within and between individuals. Various animal models have been described to study the survival patterns of these various transplanted organs (kidney, heart, lung, etc), tissues (muscle, fasciocutaneous flaps), as well as composite tissues such as an entire limb and a face^{1,2}. We describe a model for free vascularized lymph node (LN) transplantation. A simple and reproducible LN transplantation model with a low learning curve could be useful in studies concerning transplantation immunology. As opposed to non-vascularized transfer of LNs or simple lymphocyte injection studies, the model described here can provide an excellent way to elucidate the role of lymphatic system in various immunologic processes such as in transplantation tolerance, rejection and graft-versus-host disease (GVHD). Furthermore, such a model could prove to be useful in cancer metastasis studies. This article describes the transplantation of the epigastric fat pad as a means of LN transplantation using microsurgical technique in a rat model.

Since its introduction in 1967 the method of transplanting an epigastric flap that includes the overlying skin has become a standard animal model³. This procedure is mostly used to assess benefits of various drugs in flap survival patterns. In addition, it is frequently used as a model for technical exercise for teaching and maintaining expertise in performing microvascular anastomoses. The diameter of the common femoral artery is 0.8-1.0 mm and the common femoral vein measures 1.0-1.4 mm^{4,5}. The epigastric artery and vein are 0.3-0.5 mm and 0.6-0.8 mm respectively⁵. Only few studies utilizing the free vascularized epigastric fat flap without the overlying skin have been published, however all with good results in terms of feasibility⁶⁻⁸. This epigastric fat flap in rats consistently contains 1-3 LNs of a total amount of around 50 LNs⁹ and can thus be easily transplanted using microsurgical techniques by dissecting and transplanting the entire epigastric fat pad. As such, a free vascularized epigastric fat flap could be used to transplant LNs to a distant site in the same animal or to a syngeneic, allogeneic or xenogeneic animal.

MATERIALS AND METHODS

Surgical technique: Five male WF rats weighing between 200 and 350 g were used. Animals were housed in separate cages in rooms regulated in temperature (24°C), light (12 h/d), and airflow. They were fed standard rat chow and given water ad libitum. All handling of animals was per-

formed in accordance with the guidelines of the animal care and use committee of the Louisville School of Medicine and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. NIH 86-23). Also three Sprague Dowley male rats (weight between 400 and 500 g) were used and were housed in equal conditions in the Central Animal Laboratory, Utrecht University, Utrecht, The Netherlands and received care in compliance with the European Convention Guidelines. The WF animals were euthanized 150 days after treatment and the Sprague Dowley animals were sacrificed shortly after the transplantation procedure was performed.

Three epigastric fat pads were harvested from two WF donors and transplanted as vascularized flaps to the inguinal space of three WF recipients. In the three Sprague Dowley animals the left epigastric fat pad served as donor and was transplanted to the right inguinal space of the same animal. In each individual the fat pad at the recipient side was elevated, but not excised. The rats were anaesthetized, the abdominal hair shaved and the midline was marked. The epigastric fat pad was raised via a skin incision running parallel and 1-2 cm cranial to the inguinal fold. The skin was elevated and the fat pad exposed using skin retractors. The epigastric fat pad has a somewhat oval shaped outline and is oriented along the diagonal that runs from medio-caudal to latero-cranial. On the cranial side the fat pad flap is dissected of the abdominal wall using scissors and raised while the inferior epigastric and common femoral vessels are identified. Under magnification the femoral artery and vein were prepared and subsequently ligated and cut distal from the epigastric bifurcation. The epigastric nerve is resected. At the donor site the approach is similar and 1.5 cm segments of the common femoral artery and vein are cleaned of all adventitia and clamped. A longitudinal opening is created in the center of cleaned area. Using microsurgical technique the donor inguinal fat pad flap containing the LNs is connected to the recipient circulation by an end-to-side anastomosis on the femoral artery and side-to-end anastomosis on the femoral vein. The donor and recipient fat pads were positioned in the inguinal space and the skin closed with 5-0 vicryl (Figure 1 and 2).

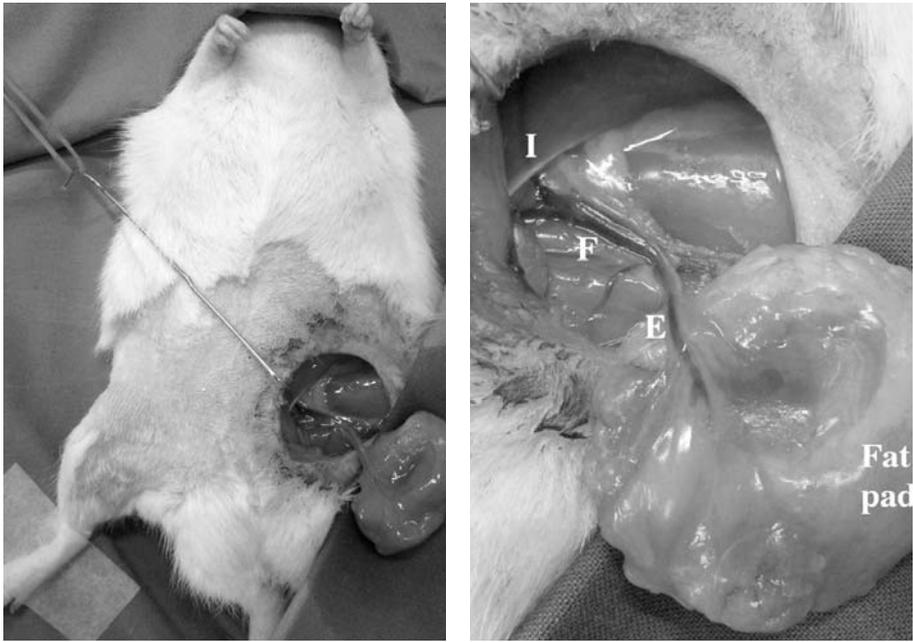


Figure 1. The epigastric fat pad of the rat dissected for transplantation. I: inguinal ligament; F: common femoral vessels; E: epigastric vessels.

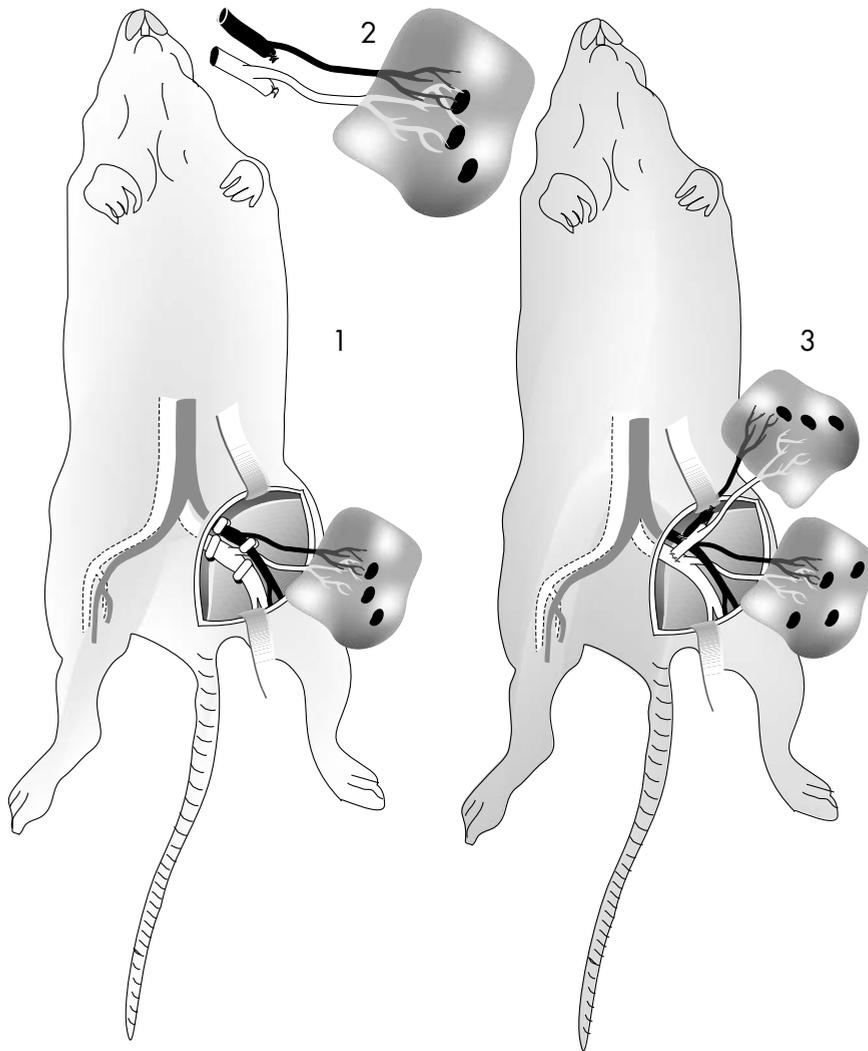


Figure 2. Schematic overview of the free microvascular lymph node transplantation in a rat model. (1) Donor rat with the epigastric fat pad prepared for transplantation. (2) The isolated fat pad containing the lymph nodes with the epigastric vessels and a segment of the common femoral vessels. (3) Recipient rat with the epigastric fat pad transplanted and anastomosed side to end on the common femoral vessels. The recipient's epigastric fat pad is not excised.

RESULTS

The pedicle arteries and veins of all transplanted LNs were patent post transplantation at the time of skin closure. All transplanted LNs of the WF animals were collected at the time of sacrifice and were found viable and with normal histologic characteristics, comparable to the popliteal and contralateral inguinal LNs, as we previously demonstrated¹⁰. The site of arterial and venous anastomosis of the Sprague Dowley animals were cut out and investigated under magnification and no obvious technical errors were seen.

DISCUSSION

LN transplantation in rats by means of epigastric fat pad transplantation can serve various purposes. Research on immunology tends to focus mainly on cell biology and little attention is paid to the structural microenvironment of secondary lymphoid organs. The described vascularized LN transplantation model could contribute to elucidate the role of the microenvironment within the LNs in transplantation tolerance, rejection, GVHD and could prove to be valuable in cancer metastasis studies. Clinically, LN transplantation could provide a tool to strengthen a locally impaired or absent immune response in patients suffering from diseases in which efficient tumor antigen presentation is weakened, such as in cancer. Furthermore, this model could be used in microsurgical training. The rat is the preferred animal for microsurgical training and access and harvesting of the epigastric flap is easy. Moreover, the length of the vascular pedicle can be varied and one can practice end-side and end-end anastomoses. Autocannibalization which is commonly seen after transplantation of anesthetic cutaneous flaps, was not seen in this model. Also, ischaemic times are better tolerated than in free muscle flaps. Shortcomings of this model would be a possible (partial) loss of the distal extremity when using an end-end anastomosis on the femoral vessels as has been observed in 2 out of 15 animals using a similar model⁶. The lack of an external flap monitor could be another disadvantage when using this LN transplantation model. LN transplantation of the inguinal fat pad as vehicle has been proven to be feasible and to provide functioning LNs at the recipient site, while transplantation of LNs without its blood supply results in fibrotic, afunctional LNs^{11,12}. Although lymphatic afferent and efferent vessels are generally not surgically anastomosed, restoration of lymphatic flow is present since lymphedema is uncommon after surgical replantation and transplantation

of tissues. Reconnection of the lymphatic afferent vessels following LN transplantation has been demonstrated to be the most effective when transplanted to a recipient site from which the LNs have been excised¹². Foster et al. demonstrated in a rat hind limb transplantation model that the lymphatic flow is restored within 12 days of limb transplantation, however he noted that the flow was shifted from the deeper lymphatics toward a more superficial pattern via the skin and subcutaneous lymphatics¹³.

We have successfully used this microvascular LN transplantation model to study the contribution of the microenvironment of lymph nodes in the induction of GVHD in chimeric rats made tolerant for the transplanted LNs¹⁰. In this study we demonstrated that transplantation of nonvascularized LNs did not lead to GVHD in tolerant recipients, whereas vascularized LNs were capable of mounting an immune response in tolerant recipients. These studies show the value of a simple and reliable LN transplantation model in rats. As such, the described LN transplantation model could be employed to increase our understanding of the role of the structural microenvironment within the LNs in various immune responses.

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Chapter 10

Vascularized lymph node transplantation induces graft-versus- host disease in chimeric hosts

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INTRODUCTION

The role of lymph nodes (LNs) in rejection of vascularized tissues has become increasingly recognized over the years. In the late 1960s, Barker et al. demonstrated that, in the absence of lymphatic drainage, allogeneic skin grafts are accepted indefinitely¹. Recently, these findings were confirmed and strengthened by a study in which mice that lacked secondary lymphoid organs (spleen, lymph nodes) were shown incapable of rejecting allogeneic skin grafts or vascularized organs². Finally, several studies have shown that tumor pathologic findings in many cases are closely related to the dysfunction or lack of a tumor's draining LNs³⁻⁵. These and similar experiments have provided strong evidence that secondary lymphoid organs are paramount in immune rejection of transplanted organs/grafts or tumors. Based on these findings, we hypothesized that adaptive immune responses could be enhanced by transplanting vascularized LNs. This hypothesis was tested in an established model for graft-versus-host disease (GVHD)⁶. In previous studies, hind limb transplantation from ACI rat donors to Wistar-Furth (WF) rat recipients that were made chimeric with ACI bone marrow (i.e. [ACI→WF]) invariably led to severe GVHD⁶. This was explained by the unidirectional immune response evoked by naive ACI cells in the limb against ACI-tolerant WF cells in the chimeric recipients. Graft-versus-host disease could be prevented by surgically removing the LNs from the hind limbs before transplantation⁷. It was concluded that the LN compartments in rat hind limbs are the main protagonists of GVHD in this model. The purpose of the current study was to establish the role of the LN microenvironment in allowing the cells contained in those LNs to establish an adaptive immune response against the recipient. Graft-versus-host responses were compared when the cells contained in the hind-limb LNs were transplanted as a cellular fraction or within their native LN microenvironment to chimeric tolerant rat recipients.

MATERIALS AND METHODS

Animals: Male, age-matched ACI (RT1-A^b) and WF (RT1-A^u) rats weighing between 200 and 350 g were used. Animals were housed in separate cages in rooms regulated in temperature (24°C), light (12 h/d), and airflow. They were fed standard rat chow and given water ad libitum. All handling of animals was performed in accordance with the guidelines of the animal care and use committee of the Louisville School of Medicine and

the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. NIH 86-23).

Groups: A total of four groups were studied. For use in groups 1, 3, and 4, chimeric [ACI→WF] recipient rats were prepared. In group 1 (n=6), cells were harvested and isolated from the LNs contained in ACI hind limbs and infused into [ACI→WF] chimeric recipients on the same day. These cells were administered in two dosages intravenously or intraperitoneally (Table 1). In group 2 (n=3), inguinal LNs were harvested from WF donors and transplanted intact as vascularized flaps to the inguinal space of WF recipients. In group 3 (n=5), inguinal LNs were harvested from ACI donors and inserted as nonvascularized grafts into the inguinal space of [ACI→WF] chimeric recipients. In group 4 (n=5), inguinal LNs were harvested from ACI donors and transplanted as vascularized flaps to the inguinal space of [ACI→WF] chimeric recipients (Table 1).

Table 1. Group disposition and treatment characteristics.

Group	No. of animals	Recipient	Donor	Procedure
1a	2	[ACI→WF] chimera	ACI	Intravenous infusion 100x10 ⁶ LN cells
1b	2	[ACI→WF] chimera	ACI	Intraperitoneal infusion 100x10 ⁶ LN cells
1c	2	[ACI→WF] chimera	ACI	Intraperitoneal infusion 300x10 ⁶ LN cells
2	3	WF	WF	Vascularized LN flap transplantation
3	5	[ACI→WF] chimera	ACI	Nonvascularized LN transfer
4	5	[ACI→WF] chimera	ACI	Vascularized LN flap transplantation

Preparation and characterization of chimeric recipients: Mixed allogeneic chimeras were prepared according to previously established protocols^{8,9}. Briefly, WF rats underwent conditioning with 950 cGy of unfractionated total body irradiation and ACI BM cells were prepared for reconstitution of the irradiated animals. Bone marrow cells were harvested

from ACI femoral and tibial bones. Aliquots of 200×10^6 unseparated cells were incubated with purified anti- $\alpha\beta$ -TCR monoclonal antibodies (MoAbs; R73; mouse immunoglobulin [Ig] G₁; BD PharMingen, Franklin Lakes, NJ) and anti- $\gamma\delta$ -TCR MoAb (V65; mouse IgG₁; BD PharMingen) for 30 minutes at 4°C. Bone marrow cells were incubated for 60 minutes at 4°C with immunomagnetic beads (Dynabeads M450; Dynal ASA, Oslo, Norway) at a bead/T-cell ratio of 20:1 and placed in a magnetic cell separator for 2 minutes to negatively select T cells. Bone marrow cells were washed, counted, and resuspended in Medium 199 (Life Technologies, Rockville, MD) plus gentamycin at a concentration of 100×10^6 BM cells per mL. Bone marrow cells were analyzed for T cells before bead depletion, after incubation with primary MoAbs, and after final depletion. Cells incubated for 30 minutes with anti- $\alpha\beta$ -TCR MoAbs (R73; mouse IgG₁; BD PharMingen), anti- $\gamma\delta$ -TCR MoAb (V65; mouse IgG₁; BD PharMingen), or rat-adsorbed goat anti-mouse IgG FITC (BD PharMingen) and washed twice after incubation were analyzed with a fluorescence-activated cell sorter. To prepare [ACI→WF] chimeras, the previously irradiated WF animals were reconstituted with 100×10^6 ACI rat BM cells (diluted in 1 mL of modified Eagle medium) via penile intravenous infusion with a sterile technique. Successful chimerism induction was confirmed 4 weeks after BM reconstitution with flow cytometry. Whole blood was collected in heparinized plastic vials and aliquots of 100 μ L were incubated with FITC-labeled anti-RT1-A^{ab} (C3; LOU/cN IgG_{2b}; BD PharMingen) and purified anti-RT1-A^u (NR3/31; rat IgG_{2a}; Serotec, Raleigh, NC) MoAb for 30 minutes. Cells were washed twice and fixed in 1% paraformaldehyde. Cells stained with purified anti-RT1-A^u were counterstained with anti-rat IgG_{2a} FITC (RG7/1.30; mouse IgG_{2b}; BD PharMingen), washed twice, and fixed. Flow typing was repeated at 60 and 90 days after BM reconstitution to confirm stable chimerism.

Lymph node cell preparation: In group 1, ACI inguinal and popliteal LNs were dissected and crushed between frosted slides in Dulbecco's Modified Eagle Medium at 4°C. Cell counts were performed, viability was assessed with use of trypan blue, and the total amount of LN cells per limb was calculated. Aliquots of 100×10^6 and 300×10^6 LN cells were prepared for infusion into [ACI→WF] chimeric recipients. Samples of LN cells were then taken to enumerate the percentage of $\alpha\beta$ TCR⁺ T and B cells. Aliquots of 1×10^6 cells were incubated with anti- $\alpha\beta$ -TCR PerCP MoAb (R73, mouse IgG₁; BD PharMingen) and anti-CD45RA PE MoAb (OX-33, mouse IgG₁; BD PharMingen) for 30 minutes and analyzed with a fluorescence-activated cell sorter. Three protocols were followed in group 1. Four of the six ani-

mals were administered 100×10^6 LN cells and the other two received 300×10^6 LN cells. Of the former four animals, two received their LN cells by intravenous administration and two by intraperitoneal administration. Both of the latter two animals received 300×10^6 LN cells.

Nonvascularized lymph node transfer: ACI donor and [ACI→WF] chimeric recipients in group 3 were anesthetized with pentobarbital 60 mg/kg and sterile surgical technique was used in all procedures. The skin was incised proximal to the midthigh area and the femoral and epigastric vessels were dissected. For donor surgery, the inguinal fat pads were isolated, the epigastric pedicles ligated, and the fat pads harvested for transfer to the recipients. For recipient surgery, the donor fat pads were subsequently inserted in the inguinal space of the recipient rats on top of the endogenous fat pads. No vascular repair was performed and the skin was closed.

Vascularized lymph node transplantation: Donors and recipients in groups 2 and 4 were prepared as described in the previous section. For donor surgery, the inguinal fat pad of each donor was isolated and a pedicle attached to the femoral artery and vein prepared (Figs. 1 and 2). The rats were then given 500 IU of heparin by penile intravenous injection followed by a 10-minute waiting period. The pedicles of the fat pads were then clamped and prepared for transplantation. For recipient surgery, the femoral vessels of the recipients were then cut at the level of the epigastric bifurcation and the proximal stump was prepared as the feeding pedicle for the vascularized lymph node flap. A microvascular anastomosis was then created to connect the vascularized donor lymph node flap to the recipient circulation.

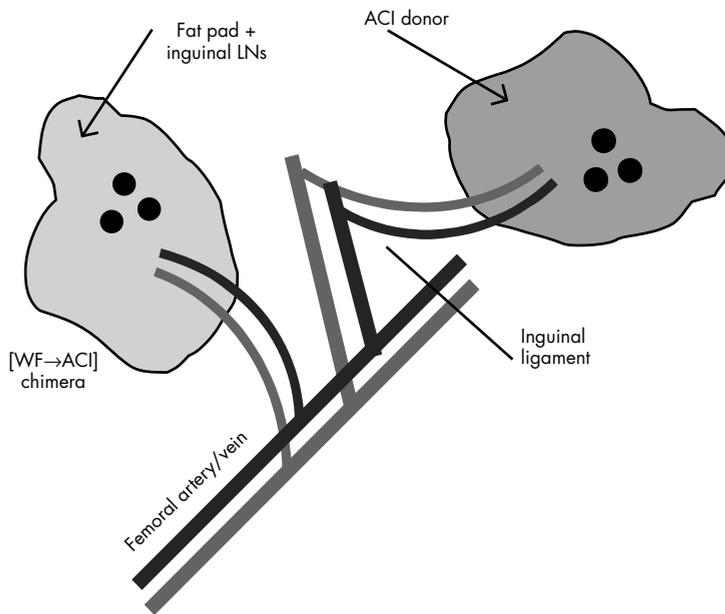


Figure 1. Vascularized lymph node flap: the ACI donor inguinal fat pad is pedicled on its femoral vessels and microanastomosed to the femoral artery and vein of the [ACI→WF] chimeric recipient.



Figure 2. Inguinal fat pad of the rat hind limb dissected for transplantation. Note the vascular pedicle of the flap (circle).

Table 2. Histopathologic grading of GVHD.

Grade	Skin	Gut	Liver
1	Basal vacuoles, lichenoid inflamed	Rare, individual crypts and apoptosis	Bile duct inflammation ± vacuolated nuclei in <25% of bile ducts
2	Grade 1 plus dyskeratosis	Contiguous crypts and apoptosis	25-50% of bile ducts involved
3	Grade 2 plus subepidermal vesicles	Crypt loss	50-75% of bile ducts involved
4	Grade 3 plus epidermal necrolysis	Denuded mucosa	>75% of bile ducts involved

Assessment of Graft-Versus-Host Disease: Graft-versus-host disease was assessed in three ways: by clinical assessment, histopathologic study, and mixed lymphocyte reaction (MLR) essays. For clinical assessment, animals were weighed every day and monitored for symptoms of GVHD. Symptoms included weight loss, dermatoreytherma, diarrhea, and nasal discharge¹⁰. Histopathologically, grading of GVHD was based on previously described criteria and included lymphocytic infiltration, epidermolysis, and bulla/cleft formation (Table 2)¹¹. Two-millimeter ear punch biopsy specimens were obtained every 28 days after LN cell infusion or LN transplantation. In addition, at the end of each experiment tongue, ear, liver, and small-intestine samples were harvested, fixed in 10% buffered formalin, and processed routinely for hematoxylin and eosin staining. At the end of each experiment, MLR assays were performed: spleens were harvested in sterile fashion and crushed with the head of a 3-mL syringe to release lymphocytes. Isolated lymphocytes were ACK-lysed, washed, and resuspended in cMLR medium (10 mL FBS, 85 mL Dulbecco's Modified Eagle Medium, 1 mL Na-pyruvate, 1 mL HEPES buffered solution, 1 mL penicillin 100 IU/mL and streptomycin 100 µg/mL, 1 mL l-glutamine 2 mM, 0.4 mL r-arginine hydrochlorate, 1 mL folic acid (1.36 mM) and l-asparagine (0.027 M); 0.2 mL 2-mercapto-ethanol (50 mM); 0.4 mL NaOH to adjust pH; 1 mL WF responder serum 1%). Cultures were incubated at 37°C in 5% CO₂ pulsed on the fourth day with 1 µCi [³H] thymidine (New England Nuclear, Boston, MA), harvested on the fifth day with an automated harvester (PHD Cell Harvester; Cambridge Technology, Cambridge, MA) and counted in a beta scintillation counter (Beckman, Palo Alto, CA). Results were expressed as counts per minute (CPMs) ± SEM.

Histologic analysis of lymph nodes: Inguinal and popliteal LNs were harvested in groups 2, 3, and 4 for histologic analysis and assessment of viability. Lymph nodes were fixed in 10% formalin buffer and processed routinely for hematoxylin and eosin staining. All tissues were read blinded by an experienced pathologist.

Criteria for euthanasia: Animals were euthanized 150 days after treatment, which was considered the end of the study. Animals with severe signs of GVHD and/or failure to thrive were euthanized when advised to do so by the veterinarians of the animal facility.

Statistical analysis: Continuous variables were expressed as means \pm SEM and experimental data were evaluated for significant differences with use of analysis of variance and the post hoc Tukey test. Differences were considered to be significant when the P value was less than 0.05.

RESULTS

Preparation and characterization of chimeric recipients: All irradiated WF hosts reconstituted with T-cell-depleted ACI rat BM cells to generate [ACI→WF] chimeras showed high levels of stable chimerism ($85\% \pm 3\%$). Chimerism levels remained stable in the groups in which chimeric recipients received ACI cells or ACI nonvascularized LNs (groups 1 and 3). In contrast, in group 4, the levels of chimerism in all animals completely changed and, by the end of the study, all hematopoietic cells were donor-derived (i.e. ACI) when assessed by fluorescence-activated cell sorter analysis ($99\% \pm 1.2\%$).

Quantification and characterization of lymph node cells: We have previously described the enumeration and localization of LN compartments in the rat hind limb⁷. Briefly, two principal locations for LNs exist: the popliteal fossa and the inguinal fat pad, in which usually a single popliteal LN and three to four small inguinal LNs can be found. In our studies, the popliteal plus inguinal LNs of a single limb contained approximately 117×10^6 cells ± 16 . The percentage of $\alpha\beta$ -TCR⁺ T cells and B cells of lymphocytes within the LNs was $61.3\% \pm 4.4\%$ and $20.6\% \pm 3.6\%$, respectively.

Development of clinical Graft-Versus-Host Disease: Weight loss is the most reliable predictor for onset and progress of acute GVHD in our rat model. Other clinical signs followed the pattern of weight loss and included erythema, nasal discharge, diarrhea, and hair loss. In group 1, 2, or 3, none of the animals developed clinical signs of acute or chronic GVHD in the course of the study (approximately 150 days). In one of the animals in group 1, which received 100×10^6 ACI LN cells by intravenous administration, notable weight loss (23%) was observed between days 24 and 32. However, the animal recovered quickly and no other clinical signs of GVHD were present, indicating that the weight loss may have been related to other causes. In group 3, one of the animals developed a local infection at the site of the surgery, which did not recover and the animal had to be killed at 119 days after nonvascularized LN transfer. However, no significant weight loss until 112 days after LN transfer was present and other signs of GVHD were not noted. In contrast, in group 4, all [ACI→WF] chimeric animals that received vascularized ACI LN transplants developed GVHD. The first signs of GVHD were present at $15.4 \text{ days} \pm 2.1$ after transplantation and all animals lost an average weight of $23.9\% \pm 4.8\%$ at $37.2 \text{ days} \pm 5.4$ after LN transplantation (Fig. 3). From 3 weeks until 9 weeks after LN transplantation, this weight change was found to be statistically significant ($P < 0.05$) compared with the control group. Three of five animals regained their initial weight at approximately day 100 and the clinical symptoms of GVHD gradually subsided. However, the other two animals died of severe GVHD: one had to be killed at day 28 and one died unexpectedly at 119 days after transplantation.

Development of histologic Graft-Versus-Host Disease: At the termination of the study in groups 1, 2, and 3, no histologic evidence of GVHD was found in any of the tissues studied. In group 4, despite the fact that clinical symptoms had largely subsided in three of five animals, all animals showed histologic evidence of GVHD at the time of termination of the study. This was most evident in the tongue, where grade 1 and 2 features of GVHD were found in all but one of the animals. The histologic results of the biopsies performed during the course of the study also confirmed our clinical findings (Fig. 4).

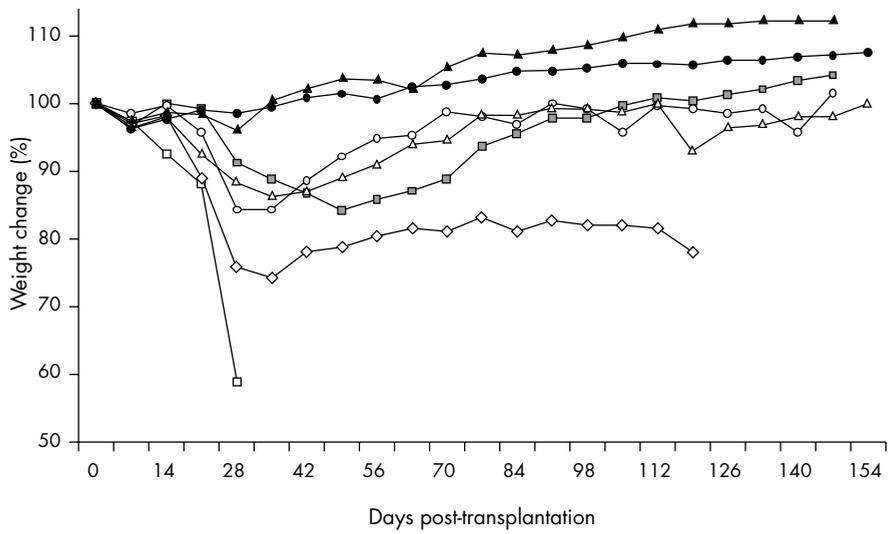


Figure 3. Weight change after transplantation: graphs from groups 2 and 3 represent averages (\pm SEM) and those from group 4 represent individual animals. Animals that received syngeneic vascularized LN flap transplants (group 2; dark circle) and [ACI \rightarrow WF] chimeras that received nonvascularized ACI LN transplants (group 3; dark triangle) showed no weight loss compared with [ACI \rightarrow WF] chimeras that received vascularized ACI LN flap transplants (group 4,—). Two of the latter five animals did not live until the study endpoint as a result of the severity of GVHD.

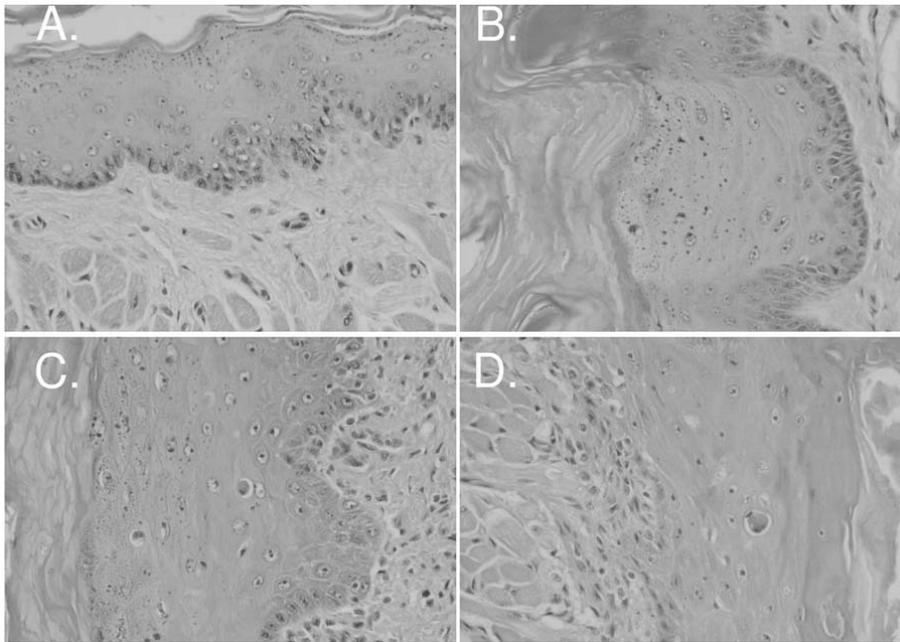


Figure 4. Representative histologic findings of the tongue samples in groups 2 (A,B) and 4 (C,D). A. Relatively normal mucosa with slight basilar vacuolization; B. Normal mucosa; C. Basilar vacuolization and dyskeratosis (grade 2); D. Lichenoid mucosal/submucosal inflammation, basilar vacuolization, and dyskeratosis (grade 2).

Graft-Versus-Host reactivity based on mixed lymphocyte

reaction: In vitro assessment established by the one-way MLR assay confirmed clinical and histologic findings. In group 4, only four of the five animals were analyzed by MLR assays, as one animal died unexpectedly of GVHD. Splenocytes from [ACI→WF] chimeras transplanted with vascularized ACI LN flaps were harvested and assessed for in vitro reactivity against splenocytes harvested from WF, ACI, and Fisher animals (i.e. third party animals). This reactivity was compared with the reactivity of WF control animals toward the same three strains. In these comparisons, the splenocytes from group 4 animals showed a significantly higher reactivity against splenocytes from WF animals (2,796 CPMs \pm 846) compared with the reactivity of WF control splenocytes against themselves (535 CPMs \pm 164; $P < 0.05$). Splenocytes from group 4 animals showed a significantly lower reactivity against splenocytes from ACI animals (674 CPMs \pm 130) compared with the reactivity of WF control splenocytes against ACI splenocytes (17,520 CPMs \pm 6,997; $P < 0.05$). This lower reactivity indicates tolerance against ACI antigens. These animals, as well as WF control ani-

mals, showed high reactivity toward third-party splenocytes (20,608 CPMs \pm 7,896 and 19,319 CPMs \pm 8,365, respectively). However, one animal that had been killed as a result of the severity of GVHD showed significantly lower reactivity against Fisher antigens.

Harvested splenocytes from chimeric animals in groups 1 and 3 showed decreased immune responsiveness against WF and ACI splenocytes and a stronger reaction to third-party (i.e. Fisher) splenocytes. This, along with the stable levels of chimerism, indicated that the circulating peripheral blood leukocytes were tolerant for WF and ACI but not for Fisher splenocytes, as would be expected from stable mixed allogeneic [ACI \rightarrow WF] chimeras. No MLR essays were performed on WF animals that received vascularized LN flaps from syngeneic animals (group 2).

Histologic analysis of lymph nodes: In group 2, all transplanted LNs were collected; in group 4, five of six transplanted LNs could be properly dissected and prepared for analysis. In group 3, transferred nonvascularized LNs were not apparent and therefore could not be analyzed histologically. It is reasonable to assume that, because of the lack of vascularization, the LNs in this group had become necrotic, and by the end of the study, had transformed into fibrotic tissue. All transplanted vascularized LNs from groups 2 and 4 were found viable and with normal histologic characteristics, similar to the popliteal and contralateral inguinal LNs (Fig. 5).

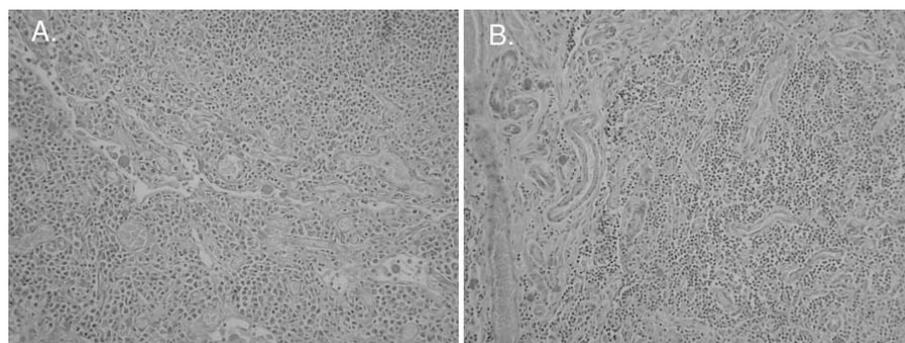


Figure 5. Transplanted LNs from groups 2 and 4 were harvested and histologically assessed for viability. All LNs studied were found viable with normal architecture and cell populations. Sections are shown from a normal control inguinal lymph node (A) and a transplanted lymph node harvested at the end of the study (B) from an animal in group 4.

DISCUSSION

The exact role of the LN microenvironment in mounting or suppressing an adaptive immune response against tumors or foreign pathogens/organs has been the subject of research for many years. Although the role of “failing” LNs has long been recognized in the pathogenesis of tumors⁵, a long-standing dogma in the field of organ transplantation holds that the immune response against primarily vascularized organ transplants does not require the presence of secondary lymphoid organs. Instead, recipient leukocytes circulating through transplanted organs and encountering foreign histocompatibility antigens on the endothelial cells in these organs were believed to initiate the immune response¹²⁻¹⁴. This dogma was recently challenged by Lakkis et al., who showed that alymphoplastic mice (i.e. mice without lymph nodes) that had undergone splenectomy and were therefore deprived of secondary lymphoid organs were incapable of rejecting cardiac allografts². In 1968, Barker et al. demonstrated that lymphatic drainage of transplanted skin grafts was paramount to immune rejection of these grafts¹.

Although these and other studies convincingly showed the importance of structural LNs to generate an effective adaptive immune response, little is known about the exact functioning of LN as organs. Because the field of immunology has an overall tendency to focus on cell biology, little attention has been paid to the structural milieu (i.e. the so-called microenvironment) of secondary lymphoid organs, especially LNs. This is perhaps best illustrated by another paradigm in transplantation immunology. The skin is considered to be the most antigenic organ of a mammalian organism. It has earned this reputation by the fact that, until recently, no immunosuppressive agents could successfully counter the immune rejection of transplanted skin grafts. In 1991, Lee et al. reported that the reason for this observation was the fact that skin is more antigenic than other organs¹⁵. Others followed this path, and the overall hypothesis became that the many minor antigens contained in the skin could explain the differential susceptibility to rejection of skin compared with other organs.

However, recently, Jones et al. showed that neither minor antigen mismatches nor the large number of antigen-presenting cells contained in the skin could explain this differential susceptibility to rejection¹⁶. In return, evidence was presented that the graft microenvironment and size could possibly explain their findings. Based on the findings presented in this article, we hypothesize that the superior lymphatic drainage of the skin could substantially contribute to its increased susceptibility to rejection. The results of this article could be explained by the LNs “giving an edge” to T cells and other

immunogenic cells to build up adaptive immune responses, at least in the model presented. Whether this is the result of the geographic collocation of dendritic cells with T cells, the highly efficient drainage and presentation of antigen, or the high concentration of growth factors and cytokines and chemokines that can be generated in this organ remains to be elucidated. However, the recognition of the intact LN as a specialized organ warrants more attention. The results of this study indicate that the ability of LNs to support B and T cells in an immune response could be dependent on the MHC identity of the LN in question. One could argue that the infused cell fractions had access to the LNs of the recipient to build up their immune response. However, even if this were the case, our data seem to indicate that it was insufficient to evoke GVHD.

In this study, in addition to adding evidence to the paramount role of the LN microenvironment, we developed a clinically feasible approach to transplanting vascularized LNs. This model could be used in a clinically relevant way to provide a strengthened immune response to patients in whom this response is locally weakened or absent. Some tumors are believed to survive because of the lack of efficient tumor antigen presentation. This is often seen in combination with the lack of draining LNs for tumors or the inefficient functioning of those that do. As such, we think vascularized LN transplantation could have potential value as a clinical treatment option for cancer.

CONCLUSION

In this study, the implications of vascularized LN transplantation to tolerant chimeric recipients were investigated. It was found that the presence of structural vascularized LNs can make a key difference when allogeneic cells are introduced into tolerant recipients. These findings stress the importance of surgical removal or consideration of LNs when performing transplantation procedures. This will become especially important as procedures for tolerance induction make their way into the clinical arena. Finally, LN transplantation could offer significant benefits in diseases in which adaptive immune responses are impaired.

ACKNOWLEDGMENTS

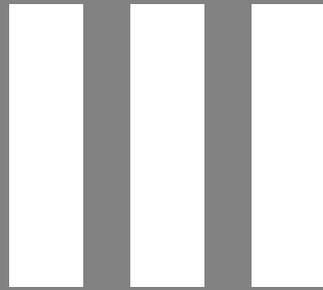
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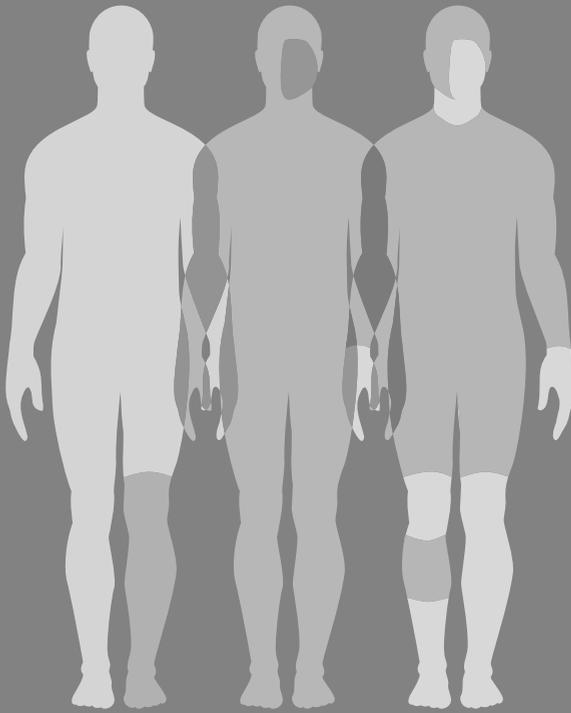
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Part





Chapter 11

Risk acceptance in composite tissue allotransplantation reconstructive procedures. Instrument design and validation

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INTRODUCTION

Composite tissue allotransplantation (CTA) has recently emerged as a new therapeutic modality to reconstruct major tissue defects to restore form and function to the head and neck region and extremities. In contrast to organ transplantation, which is a widely accepted treatment for end-stage organ failure, some argue that in CTA procedures, the risks posed by the immunosuppressant drugs outweigh the benefits received¹. Since the risk-versus-benefit ratio for CTA procedures has not yet been defined, the decision as to whether or not to perform these new reconstructive techniques is based on subjective opinions². Objective assessment of desirability and risk acceptance would certainly facilitate the decision-making process for these innovative procedures. To this end, we have developed a questionnaire-based instrument (Louisville Instrument for Transplantation [LIFT]) to objectively assess the relative risk that individuals are willing to accept in order to benefit from various CTA procedures. The goal of the work described in this paper is to validate the LIFT instrument.

MATERIALS AND METHODS

Instrument development: CTA is currently being investigated as a reconstructive modality in several clinical situations including the repair of large tissue defects involving the head and neck region and amputated extremities. Ever since the late 1990s when teams in Europe and the USA started planning to perform the first human hand transplants, a heated debate has ensued as to whether the benefits of these non-life-saving procedures justified the risks posed by the immunosuppressant drugs required to prevent graft rejection. At the center of these discussions have been issues like posttransplant quality of life and life expectancy as well as patient preference and risk acceptance for these procedures. The fundamental argument has focused on the ideological differences between the risks individuals are willing to accept to receive a "life-saving" morbidity-reducing treatment (kidney, heart, liver transplant) versus a "non-life-saving" quality-of-life-enhancing treatment (CTA procedures, such as hand transplant).

In an attempt to objectively address this question, we developed a questionnaire-based instrument to assess three forms of risk: (1) the primary risk of reduced longevity due to the toxicity of immunosuppressant drugs; (2) the risk of transplant rejection; and (3) the risk of the transplant offering inadequate functional or aesthetic results. We combined a standard gamble approach with time trade-off questions to assess individuals' acceptable

levels of risk for organ transplantation (kidney) and, ultimately, six types of CTA procedures (foot, hand, double hand, larynx, hemiface, and full face). The time trade-off technique is the preferred method for scaling health states^{3,4}. We developed a series of time trade-off questions modeled after Jalukar et al.'s work in head and neck reconstructive procedures⁵.

In time trade-off questions, subjects are asked to indicate how many years of their life, based on a fixed duration of 10 years, they would be willing to sacrifice in order to obtain the benefits of a given transplant procedure. By trading off years of life to live in a more desirable health state, an individual's total life span is reduced; consequently, an individual must sacrifice life years in order to live in a more desirable health state. To maximize the reliability of our study, we framed the questions regarding the willingness of an individual to sacrifice in six different ways. Three of the questions were framed to reflect time trade-off: (1) how many years would the individual give up in order to accept a CTA; (2) how many years would the individual need to live in order to accept a CTA; and (3) percent of remaining life that subjects would give up to receive a CTA.

The other three questions reflected a standard gamble approach. Conventionally, in the standard gamble method, individuals are asked to choose between their current health state or accept a gamble with a chance of success or failure³. Using this method, we addressed: (4) the maximum chances of rejection that the individuals would tolerate and still choose the transplant; (5) their binary consent to undergo the CTA procedure, if the chance of rejection were 50%; and (6) their dichotomous willingness to undergo the procedure given additional informed consent information on side effects of the immunosuppressive drugs and the possibility of surgical graft removal. Subjects were also asked to assess their perceived quality of life before and after transplantation, as well as the relative importance of functional and aesthetic reconstruction in their decision to undergo transplantation.

Since there are many psychological factors that may influence an individual's perception of the most desirable health state, we also assessed each subject's personality and attitudes by including measures of demographics as well as standardized psychological instruments including a self-esteem scale⁶, appearance⁷, optimism⁸, depression⁹, life satisfaction¹⁰, and questions describing the level of socially desirable responding¹¹.

Phase 1: The questionnaire was initially administered to a sample of 84 volunteer subjects (41 male, 43 female; mean age: 20 years) from the subject pool of the Department of Psychological and Brain Sciences at the

University of Louisville, KY, USA. This questionnaire consisted of 14 questions about five different transplant scenarios (foot, hand, kidney, hemiface, full face) plus 122 questions assessing body image perception, depression, self-esteem, life satisfaction, optimism, and a measure of socially desirable responding. Each transplant was specified as an injury, and photos depicting some of the deficits (foot, hand, and hemiface) with simulated CTA reconstructions were included. An image of a patient undergoing hemodialysis was also included for better understanding of this procedure. To avoid variable bias, the order of the scenarios was presented in two different formats. The average time to complete the questionnaire was approximately 60 min.

Modifications: Based on the responses obtained from the original test population, the questionnaire was modified in several ways. The combination approach of time trade-off and standard gamble questions was retained; however, to shorten the questionnaire and address several elements of difficulty with syntax and comprehension, two time trade-off questions were excluded from each transplant scenario. Those questions were not used in any analysis. Also, one time trade-off response was modified to eliminate a potential source of confusion. That did not affect the pattern of results, allowing comparisons to be made between the original and subsequent samples on five transplant scenarios. Two additional CTA scenarios were also added to the revised questionnaire; double hand transplantation and larynx transplantation. Finally, minor additions, including the time needed to complete the questionnaire and its relative difficulty, were included in the revised LIFT.

Phase 2: This questionnaire consisted of 14 different questions about the seven transplant scenarios (foot, single and double hand, kidney, larynx, hemiface, and full face) plus 100 questions based on the psychological indicators used in phase 1. In phase 2, the modified questionnaire with illustrative photos was administered to a sample population of 89 subjects (47 male, 42 female) randomly selected and individually enrolled in the study at an outpatient primary care facility. Participants received the questionnaire and basic instructions on its completion, and were asked to return their completed responses by mail. Since responses did not change significantly after the sequence of clinical scenarios had been rotated in phase 1, all phase 2 participants completed the same version of the questionnaire. The average reported time to complete the questionnaire was reduced to 45 min.

Exclusion criteria: Based on a preliminary review of responses from phase 1 and phase 2 subjects, several exclusion criteria were applied to eliminate invalid or erroneous data. First, age limitations were placed to include subjects between the ages of 18–85 years. This restriction was based on the legal age for consent for adults, as well as suitability for transplant candidate status. Responses to each transplant scenario were also evaluated, based on several criteria: (1) if more than three questions were unanswered, responses to that scenario were eliminated; (2) clearly outlying responses were eliminated based on likely misinterpretation of the question or method of responding. Based on these criteria, two subjects from phase 2 were excluded due to the age limitations, and < 1% (n = 53) of the total 6,370 transplant-related responses were eliminated from the study.

Statistical procedures: The data were subjected to four types of statistical methods. Cronbach's α coefficient is a measure of the reliability or internal consistency of a set of measurement items¹². It is calculated based on the ratio of the sum of each item's variability to the total variability across items. Fisher's F-test assesses differences between two or more groups in terms of the ratio of sum of the variance to the variance within groups. Student's t-test assesses differences between groups by the ratio of the difference between the two groups' means to the standard error of the difference¹³. Finally, Pearson's correlation r assesses the linear relation between two variables using the sum of the product of the standard scores of the two variables, divided by the number of observations¹³.

RESULTS

Of the 84 participants recruited for phase 1, an average of 82 volunteers provided complete data for the results reported below. Of the 89 participants recruited for phase 2, 66 subjects (21 male, 45 female; mean age: 50 years) returned their completed responses and were included in the study. Two subjects were excluded due to age restrictions, one for being < 18 years, the other for being > 85 years, reducing the total sample population in phase 2 to 64 participants. Responses to the clinical scenarios in the questionnaire were then pooled and compared between the 84 participants in phase 1 and the 64 phase 2 subjects. Subjects' responses were also analyzed to check for internal inconsistencies, with responses being eliminated, if the exclusion criteria were met for that transplant scenario.

Analyses revealed that six questions provided a reliable assessment of the individual's acceptance versus rejection of the risk associated with transplantation. The reliabilities of the six responses to the five transplant scenarios in phase 1 and the seven transplant scenarios in phase 2 were calculated. Responses to the years needed to live item were reflected, so that responses to all questions referred to risk acceptance. The final risk acceptance item, concerned with dichotomous willingness to undergo the procedure given additional informed consent information, was lost for the student group for the hand transplant scenario, so calculation excluded that item. Since response distributions tended to be skewed for the four questions that allowed responses on a 10-point scale, those data were subjected to logarithmic transformation. The reliability of responses to the five transplant scenarios in phase 1 ranged from Cronbach's $\alpha = 0.70$ to $\alpha = 0.83$, with average $\alpha = 0.80$. The reliability of responses to the seven transplant scenarios in phase 2 ranged from $\alpha = 0.80$ to $\alpha = 0.88$, with average $\alpha = 0.84$. Such outcomes, reported in Table 1, indicate that a consistent pattern of transplant decisions was elicited across the time trade-off and standard gamble questions for both subject groups.

Comparisons were made between the transplant decision responses of the phase 1 students and the phase 2 patients to the five transplant scenarios to which both groups responded. A multivariate analysis of variance of responses to the six questions across the five scenarios found no effect of group membership, ($F(1, 94) = 0.04$; $p < 0.84$). There was, however, an interaction of subject group with transplant scenario, ($F(4, 376) = 4.22$; $p < 0.001$). Students were more accepting than patients of the foot transplant ($t(133) = 2.11$; $p < 0.04$), and hand transplant ($t(133) = 2.50$; $p < 0.01$), but marginally less accepting of the kidney transplant ($t(133) = 1.79$; $p < 0.08$). There were no differences in the acceptance of the hemiface ($t(133) = 0.91$; $p < 0.37$), or full-face transplant ($t(133) = 0.88$; $p < 0.38$).

Measures of perceived improvements in quality of life were computed by subtracting the subject's report of the perceived quality of life before transplantation from the perceived quality of life after transplantation for each of the transplant scenarios. Comparisons were made between the perceptions of change in the quality of life of the phase 1 students and the phase 2 patients to the five transplant scenarios to which both groups responded. There was a difference between groups in such perceptions ($F(1, 113) = 15.17$; $p < 0.0001$) and an interaction between group membership and the specific organ transplant scenario ($F(4, 452) = 12.14$; $p < 0.0001$). Further analyses, presented in Table 2, revealed that the waiting room patient group perceived significantly more improvement in the quality of life

than the student group for the kidney, hemiface and full-face transplant scenarios, but not the foot or hand transplant scenarios.

Correlations were calculated between the subjects' perceptions of improvements in the quality of life from pretransplant to posttransplant and the subjects' willingness to accept each transplant. As Table 2 reports, significant correlations between perceived improvements in quality of life and transplantation acceptance were observed for students' decisions concerning the hand, hemiface and full face, and patients' decisions concerning the foot, hand, two hands, kidney, larynx, hemiface and full face. It is possible that relations were not obtained for students' decisions concerning the foot and kidney because the students perceived little change in quality of life for those organ transplants.

Correlating subjects' preferential risk acceptance for specific transplant procedures with their comments and the perceived improvement in quality of life after transplantation not only illustrated the decision-making process, but also demonstrated the construct validity of the LIFT questionnaire.

Table 1. Cronbach's α reliabilities of university student and waiting room patient groups' risk acceptance responses to transplant scenarios.

	University students (n = 82)	Waiting room patients (n = 59)
Foot	0.698	0.803
Hand	0.800 ^a	0.795
Two hands	-	0.867
Kidney	0.829	0.851
Larynx	-	0.856
Hemiface	0.814	0.877
Full face	0.829	0.864

^a missing item concerning dichotomous willingness to undergo the procedure given additional informed consent information

Table 2. Comparison of university student and waiting room patient groups' perceived improvements in quality of life, and correlations with their responses to risk tolerance questions concerning organ transplants.

	University students (n = 82)			Waiting room patients (n = 59)			
	M	SD	r	M	SD	r	t
Foot	1.831	2.251	0.082	1.968	3.016	0.475*	0.302
Hand	2.627	2.522	0.375*	2.919	3.064	0.202	0.613
Two hands		-		5.508	2.711	0.470*	-
Kidney	1.569	2.462	0.071	5.105	3.045	0.328*	6.989***
Laynx		-		3.689	2.975	0.417*	-
Hemiface	4.407	2.910	0.365*	5.661	2.975	0.311*	2.521**
Full face	4.225	2.947	0.312*	6.213	2.690	0.247*	4.171***

*p < 0.05, **p < 0.01, ***p < 0.001

DISCUSSION

CTA is an innovative surgical treatment that offers tremendous benefits over current reconstructive procedures. However, for this new treatment modality to become standard care, a clear understanding of the benefits it can provide versus the associated risks is essential. As physicians, it is our job to provide our patients with the latest and complete knowledge about the risks associated with new treatments. Ultimately, the decision to accept risk to receive the benefits of a given treatment belongs to the patient.

To objectively address this question, we have developed the LIFT instrument for assessing the amount of risk individuals are willing to accept to receive these non-life-saving, quality-of-life-enhancing procedures. Results from this questionnaire-based instrument indicate that varying levels of risk acceptance exist for the different CTA procedures. In up-coming publications, we will continue to demonstrate that the degree of risk acceptance for CTA is population-dependent, and that some groups may be willing to tolerate increased risk to undergo specific CTA to benefit from the functional and aesthetic reconstruction offered by these procedures. Most importantly, for the purposes of this study, these results indicate that the questionnaire we developed is a valid and reliable instrument to assess risk acceptance for CTA procedures.

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Chapter 12

Risk acceptance in composite tissue allotransplantation reconstructive procedures

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INTRODUCTION

In 1998, 44 years after the first human kidney transplant, the field of composite tissue allotransplantation (CTA) entered the clinical arena with the first human hand transplant procedure¹. The delay of hand transplantation's introduction into the clinical arena compared to kidney transplantation was based mainly on the lack of safe immunosuppressive agents capable of preventing rejection of skin, generally considered the most immunogenic tissue of the mammalian organism. In the 1990s, the introduction of tacrolimus and mycophenolate mofetil (MMF) solved this problem, and in 1997, preclinical studies in a large animal model demonstrated the efficacy of tacrolimus/MMF/prednisone combination therapy in preventing skin rejection, with minimal toxic side effects^{2,3,4}. Shortly thereafter, the first human hand transplants were performed by teams in Lyon, France; Louisville, KY; and Guangzhou, China^{1,5,6}.

Despite the early success demonstrated in these CTA procedures, debates among physicians and patient groups over the risk-vs.-benefit of CTA emerged and persist⁷. Critics argue that exposing transplant recipients to the risks of nonspecific immunosuppression for the benefit of nonlife-saving procedures is unjustified. The main risks associated with the use of nonspecific, lifelong immunosuppression include an increased incidence of opportunistic infections, malignancy, and end-organ toxicity⁸. On the other hand, supporters of the current immunosuppression regimen contend that because the drugs used in clinical CTA procedures are the same as those used in thousands of organ transplant recipients, the associated risks have been extensively studied, are well-known, and in select cases justify the benefits these procedures can provide.⁹ While the debate about CTA transplantation has been thoughtful, it has largely consisted of assertions by disinterested practitioners and ethicists. What has been missing has been scientific data on the views of those living with immunosuppressive medications.

Recently, based on the early success of human hand transplantation, surgical teams started contemplating performing new CTA procedures such as face transplantation. These intentions have further stirred the risk-vs.-benefit debate. In this context, the need for a scientific, objective study of the risks vs. benefits of CTA has become imperative^{9,10}.

To our knowledge, to date, no research has attempted to compare the constituents of this issue. Exactly how does the perception of individuals living with the risks of immunosuppressive drugs (organ transplant recipients) differ from that of healthy individuals not taking immunosuppressive drugs? Given that medical policies often result from the dialectic debate between various interest groups, most critical for the purpose of this study are those

who have direct experience with the risks of immunosuppressive drugs and those who do not. Gaining a deeper understanding of how these groups comprehend immunosuppression risk will facilitate bringing this ongoing argument to a satisfactory resolution.

Here, we will address this problem by presenting results based on the Louisville Instrument for Transplantation (LIFT), a questionnaire that analyzes the amount of risk individuals are willing to accept to receive different types of CTA procedures¹¹. We studied four relevant samples of both unaffected individuals (college students and randomly selected patients in a family-practice waiting room) and transplant candidates and recipients, and quantified the risk-vs.-benefit that these populations attributed to various CTA procedures, as well as kidney transplantation. While the first sample reflects the opinions of individuals who have not experienced the risks of immunosuppression (individuals not receiving immunosuppressive medication), the second population represents individuals who expect to receive immunosuppression (individuals on the waiting list to receive an organ transplant) or presently live with the risks of immunosuppression on a day-to-day basis (organ transplant recipients). Respondents were asked to estimate their perception of risk on several CTA procedures: face, hemiface, larynx, hand, double hand, and foot transplantation. The main objectives of this study were to quantitatively assess the amount of risk individuals in the above groups were willing to accept to receive different CTA procedures compared to kidney transplantation, a widely accepted procedure. To this end, three hypotheses were tested:

Hypothesis 1. Groups with and without experience with immunosuppressive drugs and transplantation will hold differing attitudes concerning risk. It is expected that those without antirejection experience will accept more risk to receive different CTA procedures (score higher on risk-acceptance measures);

Hypothesis 2. Perceptions of risk will vary by transplant procedure; and

Hypothesis 3. Kidney transplant candidates and recipients will be more likely to accept higher immunosuppressive risks for a kidney transplant than healthy controls.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Review Board at the University of Louisville.

The two primary assessment dimensions used in the LIFT questionnaire were time trade-off and the standard gamble, both of which are preferred techniques for scaling health risks^{12,13}. In time trade-off questions, subjects are asked to indicate how many years of their life, based on a fixed duration of 10 years, they would be willing to sacrifice in order to obtain the benefits of a given transplant procedure. By trading off years of life to live in a more desirable health state, an individual's total life expectancy is reduced; consequently, an individual must sacrifice life-years in order to live in a more desirable health state. In the standard-gamble method employed here, individuals are asked to choose between their current health state or accept a gamble with a specific chance of success or failure, such as a stipulated likelihood of tissue rejection.

The LIFT instrument features several transplant scenarios, in which respondents consider the time trade-off and standard-gamble possibilities. Each transplant was specified as an injury, and photos depicting some of the deficits (foot, hand, and partial face) with simulated CTA reconstructions were included. An image of a patient undergoing hemodialysis was also included for better understanding of the benefits of a kidney transplant procedure. To avoid variable bias, the order of scenarios was presented in two different formats. The average time to complete the questionnaire was approximately 60 min.

LIFT was designed to assess two forms of risk: 1) the primary risk of reduced longevity due to the toxicity of immunosuppressant drugs; and 2) the risk of transplant rejection¹¹. The first, reduced longevity, was measured by three time trade-off questions modeled after the work of Jalukar et al. in head and neck reconstructive procedures¹⁴. These items included: 1) how many years the individual would give up in order to accept a CTA; 2) how many years the individual would need to live in order to accept a CTA; and 3) percent of remaining life that subjects would give up to receive a CTA.

The second form of risk measured in this study, risk of tissue rejection, was indicated by three standard-gamble questions: 1) the maximum chances of rejection that the individual would tolerate and still choose the transplant; 2) the dichotomous willingness to undergo a CTA procedure if the chance of rejection were 50%; and 3) the dichotomous willingness to undergo the procedure, given additional informed-consent information on the side effects of the immunosuppressive drugs and the possibility of surgical graft removal.

Responses to the six questions were standardized on a 100-point scale and the mean was calculated, with a higher score indicating a greater willingness to accept risk. LIFT was determined to be a valid and reliable assessment of risk acceptance, with Cronbach's alpha averaging 0.815¹¹.

Samples: To test our hypotheses, the LIFT questionnaire was administered to four samples of individuals ranging in age from 18–85 (for a description of the study groups, see Table 1). The first two samples represented groups without immunosuppressive experience: undergraduate psychology students at the University of Louisville (n = 84) and recruits from a family-practice physician's waiting room (n = 64). The second two samples were composed of individuals who have, to varying degree, experience with immunosuppressive therapy: kidney transplant recipients (n = 42) and kidney transplant candidates currently on the waiting list to receive a donor kidney (n = 13).

The latter two groups were chosen based on the possibility that they may hold conflicting opinions about immunosuppressant drugs. Having direct exposure to the risks and side effects of these medications, kidney transplant recipients were expected to be more realistic and possibly skeptical about the risks of immunosuppressive therapy. Conversely, kidney transplant candidates who are likely eager, perhaps even desperate, to undergo renal surgery are likely to have a more idealized assessment of the drugs or be disposed to minimize the risks of immunosuppression and rejection, particularly for a kidney transplant.

Table 1. Demographic characteristics of four samples.

	Students (n = 84)	Patient controls (n = 64)	Transplant recipients (n = 42)	Transplant candidates (n = 13)
Sex				
Male	49%	32%	50%	62%
Female	51	68	50	38
Age (mean)	20	50	45	45
Ethnicity				
White	76%	95%	86%	85%
Black	16	0	14	15
Others	8	5	0	0
Marital status				
Never Married	87%	11%	19%	15%
Married	2	69	57	69
Cohabiting	11	2	2	0
Separated	0	2	2	0
Divorced	0	11	19	0
Widowed	0	5	0	15
Education				
Less than high school	0%	14%	12%	23%
High school	11	34	31	23
Some college	81	33	31	39
College degree	7	17	25	15
Household income				
Under \$20,000	15%	16%	28%	15%
\$20,000-\$40,000	17	33	31	38
\$40,000-\$60,000	14	23	17	31
\$60,000-\$80,000	21	8	5	0
Over \$80,000	30	14	12	15

Table 2. Risk acceptance of seven categories of transplantation by four samples*.

		All cases (n = 203)^a	Student controls (n = 84)	Patient controls (n = 64)	Kidney Tx recipients (n = 42)	Kidney Tx candidates (n = 13)
Foot	M	23.042 _a	23.130	19.002	26.442	31.401
	SD	21.026	23.378	19.596	21.129	23.378
Group Difference	F	1.863				
	P	0.137				
Hand	M	32.361 _b	33.230 _a	26.889 _b	39.026 _a	32.150 _a
	SD	20.201	17.767	21.211	20.840	22.301
Group Difference	F	3.247				
	P	0.023				
Larynx	M	38.644 _c		34.092 _a	41.354 _a	52.299 _b
	SD	38.644		23.529	19.241	22.070
Group Difference	F	4.211				
	P	0.017				
Kidney	M	42.902 _d	34.292 _a	45.231 _b	53.150 _c	53.963 _c
	SD	22.497	23.982	21.648	15.345	15.138
Group Difference	F	9.303				
	P	0.000				
Two-hand	M	47.335 _d		47.022	46.907	51.172
	SD	21.343		22.568	20.191	19.886
Group Difference	F	0.258				
	P	0.855				
Hemiface	M	55.095 _e	54.710	53.246	57.982	57.362
	SD	23.090	24.390	24.375	17.772	24.647
Group Difference	F	0.402				
	P	0.752				
Face	M	61.341 _f	63.357	59.979	59.297	61.613
	SD	22.705	24.157	23.080	20.351	19.417
Group Difference	F	0.408				
	P	0.747				

*Means (M) in left column with different subscripts, or in same row with different subscripts, differ $P < 0.05$. F-tests were conducted to determine whether reliable differences existed between subject groups within transplant category. The F test statistics and associated P values are reported below the risk acceptance means for all cases. When the F-tests were significant, t-tests were conducted to evaluate the difference between adjacent means. The t-test statistics and associated P values are reported in the text. Means in the left column with different subscripts, or in the same row with different subscripts, differ $P < 0.05$. For example, if one mean has the subscript "a" and another mean has the subscript "b" then the two means are significantly different. A third mean that has the subscript "ab" does not significantly differ from either of those two means. ^an = 203, except larynx and two-hand transplant, for which n = 119.

Role of funding sources: The study sponsors had no role in the study design; collection, analysis, or interpretation of data; or writing of the manuscript.

RESULTS

A repeated-measures multivariate analysis of variance was conducted to examine the level of risk acceptance for the five categories of transplantation (foot, hand, kidney, hemiface, and face) for which responses were solicited from all four groups of research participants (student controls, patient controls, kidney transplant recipients, and kidney transplant candidates). This analysis found significant differences in research participants' risk acceptance of different transplant procedures ($F(4,796) = 94.78, P < 0.0001$), but no significant difference between groups in risk acceptance ($F(3,199) = 2.070, ns$). These findings, however, were qualified by a significant transplant type by group interaction ($F(12,796) = 3.61, P < 0.0001$), which will be explained below. The order in which scenarios were presented did not affect the results of LIFT.

Similar results were obtained when analyses were conducted to examine the level of risk acceptance of seven categories of transplantation (foot, hand, two hands, kidney, larynx, hemiface, and face) for which responses were solicited from three groups of research participants (patient controls, kidney transplant recipients, and kidney transplant candidates). Again, there were no significant statistical differences between groups in risk acceptance ($F(2,116) = 2.69, ns$), but significant differences in research participants' risk acceptance of different transplant procedures ($F(6,696) = 43.35, P < 0.0001$). These results fail to confirm hypothesis 1, which stated the expectation that groups would differ in risk acceptance, and suggest that the research groups perceived risk similarly. Hypothesis 2, however, was partially supported. There was considerable variation in risk acceptance by transplant procedure. The transplant type by group interaction ($F(12,696) = 1.53, P < 0.11$) was not statistically significant. These results indicated that the four groups of research participants generally accepted comparable levels of risk overall, but there were notable differences in the amount of risk that would be accepted for specific procedures.

These results indicate that the four groups of research participants generally accepted comparable levels of risk overall, but there were notable differences in the amount of risk that would be accepted for specific procedures. Paired t-tests were conducted between the levels of risk acceptance for each

procedure, and resulted in a ranking of risk acceptability (Table 2). Across samples, risks were ranked as follows: the highest level of risk was accepted for a full face transplant, which was greater than that for a hemiface ($t(222) = 6.22, P < 0.0001$); risk acceptance for hemiface was greater than that for transplantation of two hands ($t(119) = 3.89, P < 0.0001$); risk acceptance for hemiface was also greater than that for a kidney transplant ($t(222) = 6.63, P < 0.0001$); risk acceptance for a kidney transplant was equivalent to that for a double hand transplant ($t(118) = 0.78, ns$); and risk acceptance for a double hand transplant was greater than that for a larynx transplant ($t(118) = 4.37, P < 0.001$), which was greater than that for a single hand transplant ($t(118) = 3.24, P < 0.002$). Finally, risk acceptance for a hand transplant was significantly greater than that for a foot transplant ($t(118) = 3.24, P < 0.002$).

The transplant by group interaction was explored by conducting post hoc Tukey analyses on group differences in risk acceptance for each transplant type. The primary finding was that, as expected, kidney transplant recipients and candidates were both willing to accept more risk for a kidney transplant than were the control groups. Hypothesis 3 is therefore supported by these data. Kidney transplant candidates and recipients did not differ in regard to perception of risk of kidney transplantation, contrary to expectations.

Other findings of interest were that patient controls (waiting-room patients) were less willing than the other groups, who did not differ, to accept the risks of a hand transplant, and patient controls and kidney transplant recipients were less willing than kidney transplant candidates to accept the risks of a larynx transplant.

DISCUSSION

This study sought to test the hypothesis that individuals with no direct experience with immunosuppressive drugs would have a different perception of risk than kidney transplant recipients with present or expected experience with the medications. Contrary to our prediction, this study found that the viewpoint of those who live with the risks of immunosuppressant medications does not differ from that of those who do not. In other words, people with and without direct immunosuppressant experience report similar conclusions about risk. Of special note, however, is that transplant recipients, who live with the risks of immunosuppression, are more likely to accept risk than healthy individuals ($F(1, 124) = 3.65, P < 0.05$).

Individuals' views of risk differ widely. Outside of medicine, for example, many people eagerly engage in sports such as motorcycle racing, mountain climbing, polo, or skydiving that others deem dangerous and foreboding of catastrophic ends. Although others do not, sports enthusiasts clearly see the benefits derived from participating in these activities as worth the risks inherent in them. Within medicine as well, individuals view risks differently. Thus it is difficult if not impossible to ascribe an absolute value to the risk/benefit ratio of various transplantation procedures. It is possible, however, to compare the risks/benefits of different types of transplant procedures, and to provide a relative assessment or ranking for a particular group of procedures.

To determine the ranking of acceptances assigned to the transplant procedures in the LIFT questionnaire, we used psychologically accepted dimensions (i.e., time trade-off and standard gamble) for various procedures, and used the appropriate statistical analyses to compare the preferences for each of them. In establishing this relative framework of acceptance, it was important to include a standard that could be considered a reasonable benefit that warrants the risks of immunosuppression. We chose kidney transplantation as our baseline, because it is a treatment in which the risks of immunosuppression are widely accepted as being worth the benefits of one of these procedures. Unlike other "life-saving" organ-transplant procedures, kidney transplantation is arguably more like CTA procedures, in that it is also a nonlife-saving procedure. That is, a kidney transplant is often seen as desirable, but people can survive on hemodialysis.

Remarkably, all groups ranked the transplant procedures (face, hemiface, larynx, hand, double hand, and foot transplantation) in the same order of acceptance, and hence the same order of risk-vs.-benefit. The greatest risk acceptance was for transplantation of the face, followed by the hemiface, and then significantly lower for two hands and kidney, followed by larynx, then significantly lower for a single hand, and then significantly less for the foot.

Although we found that each group ranked the seven procedures in the same order of acceptance, there were minor differences in acceptance of each procedure individually by the four populations. Probably the most important difference was that kidney transplant recipients and individuals waiting to get kidney transplants assigned the same benefit to kidney transplantation, which was higher than that assigned to kidney transplantation by the two control groups. Individuals with the need for a kidney transplant place higher value on that procedure, by reporting greater risk acceptance, than individuals without that need. This is a critical finding, because it indi-

cates that real-life exposure to immunosuppression does not alter the value or “benefit” that recipients attribute to their transplant. This result also serves as a counterargument to the misperception that transplant candidates, even when given informed-consent information, are unable to realistically assess and understand the risks of nonspecific lifelong immunosuppression. In this study, the perceptions and judgments of transplant candidates were almost identical to those of transplant recipients. Consequently, the views of both transplant candidates and recipients might be accorded special weight when evaluating the risk/benefit ratio of new transplant procedures.

CONCLUSIONS

This study provides indirect evidence that transplant recipients understand the risks of immunosuppression that they will be exposed to once they receive a CTA procedure. Both kidney transplant recipients and those on the waiting list to receive a kidney attributed the same high benefit to kidney transplantation, suggesting that transplant recipients understand the risks of immunosuppression prior to transplantation.

The results of this study also show that the benefits assigned to, and therefore the risks accepted for, two hypothetical (face and hemiface) and one established but controversial (double hand) CTA procedures are at least as high as for the well-established and generally accepted procedure of kidney transplantation. This means that subjects perceive the potential benefits of these CTA procedures, in terms of improved aesthetics and function, to be so valuable that they outweigh the immunosuppression-related risks and harm. It is noteworthy that kidney transplant recipients, who live with the risks of immunosuppressive drugs, attribute greater benefit to a face transplant than a kidney transplant. Based on these findings, we conclude that certain CTA procedures, such as face and larynx transplants, convey benefits to a recipient that are perceived by subjects, including highly experienced subjects, to warrant the risks of these procedures.

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Chapter 13

Investigation of risk acceptance in facial transplantation

PLASTIC RECONSTRUCTIVE SURGERY 2006; 118(3): 663-70

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INTRODUCTION

On December 23, 1954, a team of doctors in Boston, Massachusetts, led by Dr. Joseph Murray, a plastic surgeon, transplanted a kidney into a dying 23-year-old man in the first successful long-term transplant of a human organ. At the time, this revolutionary medical advancement was hailed by some as “a miracle of medicine,” while others accused Murray and his team of “playing God,” saying that the surgery should not have been done¹. Ethical debate has accompanied medical advancements throughout history, and this is particularly true for transplantation medicine. In the 50 years that have passed since that first successful kidney transplant, organ transplantation has saved and improved the lives of well over 400,000 people in the United States alone (United Network for Organ Sharing, at <http://www.unos.org>), and Murray’s work is celebrated today as one of the greatest advancements in modern medicine.

The recent introduction of composite tissue allotransplantation, particularly in the form of hand and facial transplantation, has served to rekindle many of the same topics of debate that have accompanied transplantation since its beginning, and has generated new controversies as well. Of primary interest in the present study is the ethical debate focusing on risk versus benefit, which has accompanied transplantation medicine from its outset. The ethical question can be specifically posed in the following way: Do the risks posed by the life-long immunosuppression required to prevent rejection justify the benefit of receiving a new face? In this “risk-versus-benefit” debate, proponents claim that the benefits outweigh the risks, while critics claim they do not.

Proponents argue that, in a select number of severely disfigured individuals, facial transplantation can provide better functional and aesthetic outcomes than conventional reconstructive methods, and in doing so, it can improve the quality of life of affected individuals^{2,3}. While critics concede that the risks associated with surgically transplanting facial tissues are essentially the same as those present in conventional reconstructive procedures, their primary argument against proceeding with clinical facial transplantation centers on the risks of immunosuppression and rejection. Specifically, their main concern focuses on the risks posed by the life-long immunosuppression that recipients would require to prevent rejection of the transplanted facial tissues and the risks associated with facial tissue rejection itself^{4–12}.

Proponents and critics alike have based their positions largely on theoretical discussions and subjective opinions. None have referred to the direct life experiences of those confronting the risks of immunosuppression or

have collected data from individuals who might benefit from a face transplant. Decisions of risk must include input from those taking the risk, because, as research shows, perception of risk and the amount of risk individuals are willing to accept in day-to-day life differ widely. This difference is largely due to the different life experiences to which each individual is exposed. Edgell et al. applied a risk-versus-benefit equation to hand transplantation and found that people from varied backgrounds see hand transplantation from different perspectives and therefore have dissimilar opinions on how desirable a hand transplant is¹³. Not surprisingly, it is difficult if not impossible to ascribe an absolute value to the risk/benefit ratio of various transplantation procedures. It is possible, however, to compare the risks and benefits of different types of transplant procedures and to provide a relative assessment or ranking for a particular group of procedures.

After this theme, previous research on risk assessment has shown that people receiving immunosuppression treatments or people who are candidates for medical treatment requiring immunosuppression report high levels of risk acceptance. Studies comparing healthy controls to hand amputees (who could benefit from a hand transplant) and kidney transplant recipients (who live with the risks of immunosuppression) have shown few differences in willingness to accept risk¹⁴. Other studies, which are perhaps more applicable to the present article, have found that healthy controls and kidney transplant recipients (who live with the risks of immunosuppression) would accept higher degrees of risk to receive a face transplant than a kidney transplant¹⁵.

The research reported on in this article extends this line of study by investigating perceptions of risk acceptance among a sample of facially disfigured individuals. To our knowledge, there have been no quantitative studies that assess "real world" decisions made by disfigured individuals who could directly benefit from a facial transplant procedure. Given this gap in the literature, we designed and conducted the present study to evaluate the degree of risk that individuals who could directly benefit from facial transplantation are willing to accept to receive this new treatment.

MATERIALS AND METHODS

To determine the amount of risk individuals are willing to accept to receive various transplant procedures, we developed and validated a questionnaire-based instrument, the Louisville Instrument for Transplantation, or LIFT, in which we used psychologically accepted dimensions for different trans-

plant procedures and used the appropriate statistical analyses to compare the preferences for each of them. Questions assessing body image perception, depression, self-esteem, optimism, socially desirable responding, and demographics were also included, although they are outside the scope of this report¹⁶.

We structured the questions to assess two primary categories of risk: risks posed by the immunosuppression a recipient would have to take and the risks associated with rejection of the facial tissues. In establishing this relative framework of acceptance, it was important to include a standard that could be considered a reasonable benefit that warrants the risks of immunosuppression. The two primary assessment dimensions used in the questionnaire were time trade-off and the standard gamble, both of which are commonly used techniques for scaling health risks^{17,18}.

In time trade-off questions, subjects are asked to indicate how many years of life, based on a fixed duration of 10 years, they would be willing to sacrifice to obtain the benefits of a given transplant procedure. By trading off years of life to live in a more desirable health state, an individual's total life expectancy is reduced; consequently, an individual must sacrifice life-years to live in a more desirable health state. In the standard gamble method utilized here, individuals are asked to choose their current health state or accept a gamble with a specific chance of success or failure, such as a stipulated likelihood of tissue rejection.

The Louisville Instrument for Transplantation features several transplant scenarios for which respondents consider the time trade-off and standard gamble possibilities. Each of the transplant scenarios (foot, single hand, double hand, larynx, kidney, hemiface, and full face) was specified as an injury, and three deficits, foot, hand, and hemi-face amputations, were illustrated in photographs along side pictures of the respective simulated transplant reconstructions. An image of a patient undergoing hemodialysis was also included for a better understanding of the benefit of a kidney transplant procedure. Each transplant decision in the Louisville Instrument for Transplantation began with a brief but realistic medical scenario. For example, the kidney transplant situation was presented as follows: *You sustained an injury that destroyed both of your kidneys. You currently receive dialysis three times per week, for 3 hours per session. You tolerate dialysis reasonably well, although it is uncomfortable, time consuming, and limits travel. (Please see Fig. 2 in photo booklet for picture of renal dialysis patient.) Similarly, the hemiface injury was presented in comparable terms: You sustained an injury that destroyed the left half of your face. Tissues from other areas of your body have been used to cover the defect. After*

this treatment, the skin looks tight and blotchy, and you lack your left eyebrows, lips, and most of your nose. People have difficulty looking at you. (Please see Fig. 4 in photo booklet for picture of individual with face injury.)

To avoid variable bias, the order of the different scenarios was presented in two different formats. The average time to complete the questionnaire was approximately 60 minutes.

We included kidney transplantation as our baseline, because unlike other "life-saving" organ transplant procedures, renal transplantation is arguably more akin to composite tissue allotransplantation techniques in that both are "quality-of-life-improving" surgeries. That is, a kidney transplant is commonly seen as desirable, but people can survive on hemodialysis. Also, kidney transplantation is a standard treatment in which the risks of immunosuppression are widely accepted as being worth the benefits.

The first form of risk used in this study, reduced longevity due to the toxicity of immunosuppressant drugs, was measured by three time trade-off questions modeled after Jalukar et al.'s work in head and neck reconstructive procedures¹⁹. These items included (a) the number of years individuals would give up to accept a given transplant procedure; (b) the number of years individuals would need to live to accept a given transplant procedure; and (c) the percentage of remaining life that subjects would give up to receive a given transplant procedure. Risk acceptance, when considering the need to take life-long immunosuppression medication, was framed in terms of respondents' willingness to undergo the transplant given information taken from an informed consent document describing²⁰ specific side effects.

The second form of risk, risk of tissue rejection, was indicated by three standard gamble questions: (a) the maximum chances of rejection that the individual would tolerate and still choose the transplant; (b) respondents' dichotomous willingness to undergo the composite tissue allotransplantation procedure with a specified risk of rejection ("*If the chance of rejecting the full face were 50 percent in the first year, would you still get the transplant?*"); and (c) respondents' dichotomous willingness to undergo the procedure given informed consent information on the side effects of the immunosuppressive drugs and the possibility of surgical graft removal ("*After your transplant, you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or vi-*

ral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life-threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed. After reading the above, would you still want to get the transplant?”). Responses to these questions were equated on a 100-point scale and the mean was calculated, with a higher score indicating a greater willingness to accept risk. The Louisville Instrument for Transplantation questionnaire was determined to be a valid and reliable assessment of risk acceptance; Cronbach’s alphas averaged 0.815 in the validation sample and 0.784 in the present sample.

Statistical analysis: A multivariate analysis of variance allowed determination of the effect of the three patient populations (healthy controls, kidney transplant recipients, and facially disfigured individuals) on risk acceptance for the seven transplant procedures (foot, single hand, double hand, larynx, kidney, hemiface, and full face), while controlling for overall variation in risk acceptance for the different procedures. Univariate analyses of variance were used to test of the effect of the type of transplant procedure and the interaction of type of procedure and group membership. Student t tests were utilized to contrast the mean risk acceptance between two designated groups. Chi-square analysis was used to contrast two frequencies.

Samples: The questionnaire was administered to three samples of individuals ranging in age from 18 to 85 years with differing life experiences vis-à-vis transplantation and disfigurement. The first sample (n = 150; mean age, 32.73 years) consisted of controls who had no direct experience with the risks and benefits associated with facial transplantation. They included undergraduate psychology students at the University of Louisville (n 84) and recruits from a primary care physician’s waiting room (n = 66). The second sample was composed of kidney transplant recipients (n = 42; mean age, 45.15 years). These individuals have direct experience and live with the risks of immunosuppressive therapy. The third group involved individuals with facial disfigurement (n = 34; mean age, 51.88 years). Their facial deformity ranged from 2 to 100 percent of the face (mean, 31 percent) showing disfigurement. These individuals could be said to have direct experience with the possible benefits that facial transplantation could provide.

RESULTS

Risks of immunosuppression: When considering 20 immunosuppression-related potential side effects for the kidney transplant, 61 percent of normal respondents, 100 percent of kidney transplant recipients, and 85 percent of the facially disfigured respondents were willing to undergo a kidney transplant. The differences among these groups were significant ($\chi^2(2) = 26.43$, $p < 0.0001$) (the number in parentheses represents degrees of freedom). Considering the same 20 immunosuppression-related risks, 86 percent of normal respondents, 93 percent of kidney transplant recipients, and 77 percent of the facially disfigured respondents were willing to undergo a face transplant procedure. The differences among these groups were significant ($\chi^2(2) = 4.25$, $p < 0.12$).

Risks of rejection: When asked, "What would be the maximum chances of rejection you would tolerate and still get a kidney transplant," the mean for the control respondents was 35.95 percent, which indicated less risk acceptance than the mean for the kidney transplant recipients, which was 52.68 percent ($t(187) = 3.26$, $p < 0.001$). The maximum chance of rejection for a kidney transplant accepted by the facially disfigured respondents was 40 percent, which was not significantly different from that for the kidney transplant recipients ($t(73) = 1.86$, $p < 0.07$) or the controls ($t(180) = 0.84$, $p < 0.40$).

In response to the question "What would be the maximum chances of rejection you would tolerate and still get a face transplant?," the controls' mean was 51.34 percent, which did not significantly differ from the risk acceptance of the mean for the kidney transplant recipients of 56.00 percent ($t(180) = 0.88$, $p < 0.38$). The maximum chance of rejection for a face transplant accepted by the facially disfigured respondents was 35.29 percent, which was less accepting of risk than that for the kidney transplant recipients ($t(72) = 2.96$, $p < 0.004$) or the respondents in the control group ($t(174) = 2.80$, $p < 0.006$).

When asked, "If the chance of rejecting a transplanted kidney were 50 percent in the first year, would you still get the transplant?," 59 percent of controls respondents, 88 percent of kidney transplant recipients, and 77 percent of facially disfigured respondents were willing to undergo the procedure under such circumstances.

Applying the same conditions to face transplantation, 87 percent of controls, 88 percent of kidney transplant recipients, and 71 percent of facially disfigured respondents were willing to undergo the procedure.

Comparing different transplant procedures: Multivariate analyses of variance, as shown in Table 1, revealed that respondents in all three populations (healthy controls, kidney transplant recipients, and facially disfigured individuals) would accept different amounts of risk for the seven different types of transplant procedures (foot, single hand, double hand, larynx, kidney, hemiface, and full face) ($F(6,1320) = 81.41, p < 0.0001$). All differences in risk acceptance among the various types of transplant procedures were significant, except for one. Respondents would accept the least amount of risk to receive a foot transplant, followed by significantly more risk for a single hand transplant ($t(222) = 7.64, p < 0.0001$), followed by a larynx transplant ($t(222) = 5.48, p < 0.0001$), and then a kidney transplant ($t(222) = 2.95, p < 0.01$). Risk acceptance for a kidney did not differ significantly from that for two hands ($t(222) 0.95, p 0.95$). Risk acceptance both for a kidney ($t(222) = 5.97, p < 0.0001$) and for two hands ($t(222) = 5.85, p < 0.0001$) was lower than that for a hemiface. Finally, risk acceptance for a hemiface transplant was lower than for a full face transplant ($t(222) = 6.15, p < 0.0001$). Thus, respondents were willing to accept the most risk for a full face transplant, more than the risk for any of the other six transplant procedures.

Table 1. The relative ranking from high to low of risk acceptance by transplant procedure.

Transplant Procedure	t(222)	p	SD*
1st: Full face			24.055
2nd: Hemiface	6.15	< 0.0001	23.572
3rd: Kidney	5.97	< 0.0001	22.193
4th: Two hands	5.85	< 0.0001	18.391
5th: Larynx	5.95	< 0.01	18.186
6th: Single hand	5.48	< 0.0001	20.692
7th: Foot	7.64	< 0.0001	20.765

*Data for standard deviation (SD) were selected according to group 5 > 0.

DISCUSSION

In sum, all three groups were willing to accept more risk for a face transplant than for any of the other six transplant procedures (Table 2). Of note

here is that the three groups would accept significantly more risk for a hemiface and full face transplant than for a kidney transplant, although the latter is a procedure considered to be standard care in which the risks versus benefit are not debated.

Our findings suggest that patients who have direct experience with (i.e., live with) the risks of immunosuppression and rejection (kidney transplant recipients) or who could benefit from a face transplant (facially disfigured individuals) view the risks of immunosuppression and rejection significantly different from the way unaffected individuals (controls) do. This difference in views leads to different risk/benefit assessments, and there exists no way to determine which assessment is the more accurate one. The acceptance rate of a face transplant by kidney recipients might have been higher than that for others because the kidney recipients were already taking antirejection medications. The lower acceptance rate among disfigured respondents may be somewhat misleading. Research has shown that some facially disfigured individuals adjust to their condition quite well and integrate their appearance into their lifestyles. When this occurs, they adapt to their disfigured face to the point that they prefer their new appearance and would refuse the opportunity to have surgical changes made to their faces^{20,21}.

Hence, the dispute between these different assessments can be resolved only by ranking one of the frames of reference over the other. As we have said, however, there seem to be no criteria by which one could judge one view "more rational" than the other or by which one could rank the different views in terms of their "greater or lesser rationality." Hence, we believe that the ranking must be an ethical one. We appeal to the cardinal ethical principle of "respect for persons" or "respect for autonomy" to do this, and therefore we maintain that the patient should choose^{2,3}.

As teams around the world prepare to perform human facial transplantation, critics contend that the risks posed by the life-long immunosuppression that face recipients would require to prevent tissue rejection and the risks posed by rejection itself do not justify the benefits of this procedure. This sentiment related to the risks posed by immunosuppression is represented, for example, in the Royal College of Surgeons' Working Party Report on Facial Transplantation. This report states, "The need for lifetime immunosuppression carries considerable long-term risks which appear to outweigh any premature attempt to open the gates to facial transplantation . . . until there is further research and the prospect of better control of these complications (immunosuppression related) it would be unwise to proceed with human facial transplantation"⁴. In reference to the risks associated with rejection of facial tissues, Caplan states, "If the procedure should result in acute re-

jection then the subject may die with the entire graft sloughing off of his or her head”⁷.

These critics are opposed to moving facial transplantation research into a clinical phase, asserting that such procedures should not be performed until advances in transplant immunology make it possible to reduce or eliminate the risks. While the critics understand that an improvement in the disfigured person’s quality of life could probably be gained from a successful face transplant, they view facial transplantation as subjecting patients to risks that do not justify these benefits.

Risk/benefit assessments are central to ethical decisions in surgical/medical research. Our study provides an evidential basis with which facially disfigured patients might be invited to participate as subjects in such research. Our data show that, of the subjects we surveyed, all three groups were willing to accept more risk for a face transplant than for any of the other six transplant procedures, namely, foot, single hand, double hand, larynx, kidney, and hemi-face transplants. This indicates that the risks of a face transplant, when fully understood by subjects, prove to be acceptable to them. That is to say, when informed of the risks of immunosuppression and the risks of rejection, a significant majority of the subjects would choose to undergo the procedure. Indeed, when considering a stipulated (although unrealistically high) 50 percent possibility of rejecting the transplanted facial tissue within 1 year, 71 percent of the facially disfigured persons, 88 percent of organ recipients, and 87 percent of nonaffected individuals were willing to undergo the procedure. In addition, when confronted with a list of 20 known immunosuppression-related potential side effects, 77 percent of the facially disfigured respondents, 93 percent of kidney transplant recipients, and 86 percent of the control respondents were willing to undergo a face transplant procedure.

Our findings indicate that facially disfigured individuals view the prospect of a face transplant differently than do critics of the procedure, such as the Royal College of Surgeons’ Working Party. Facially disfigured persons may perceive the choice of not having the transplant as equivalent to their willing acceptance of a terrible loss, namely, the loss of a normal facial appearance and of the quality of life that such a normal appearance afforded. Such voluntary acceptance of the loss proves immensely difficult, because they know first hand the misery involved in their present disfigurement. They perceive the face transplant, on the other hand, as an opportunity – even with its admitted risks – of returning to a normal appearance such as the one they previously enjoyed. Given this choice, patients find it extremely difficult to voluntarily acquiesce to the life-long loss. They are thus

willing to run serious risks to have the chance to return to their earlier status quo, namely, a normal facial appearance and the quality of life that it affords.

We suggest that the most fruitful way to conceive of the difference between subjects' assessment of the risk/benefit ratio and the critics' assessment of it lies in viewing the two groups as viewing the risks and benefits from within different "frames of reference." Using different frames of reference, two groups may evaluate the utilities of the same procedure differently¹³. Accordingly, two groups of people can examine the same options and weigh them quite differently, although given the frame of reference that each group uses, the weightings are each entirely rational. From within the frame of reference of the critics of facial transplantation, it is rational to forgo the transplant because the risks that it entails outweigh the benefits to be gained. From within the disfigured person's frame of reference, on the other hand, the opportunity to eradicate the devastating loss from which he or she suffers daily and return to the comfortable quality of life afforded by a normal facial appearance outweighs the risks involved. Both weightings are rational, given the different frames of reference, despite the fact they entail different conclusions.

If our interpretation of the differences between the risk/benefit assessments given by the subjects in our survey, on the one hand, and by critics of facial transplantation, on the other, is accurate, it is not likely that any resolution of these differences can be attained by a careful re-examination of the risks and benefits of the procedure. The dilemma arises after all from the different frames of reference within which the same risks and benefits are being assessed. The resolution may lie then in ranking one frame of reference as higher than the other, even while we remain fully cognizant of the "rational" legitimacy of each. It is a cardinal ethical principle of health care research that "respect for persons" entails that the fully informed, voluntary choices of research subjects should be respected. We fail to see, then, why the frame of reference of the critics of the procedure should carry greater weight than the frame of reference of informed subjects who act voluntarily. We think as a result that it is ethically justified to move facial transplantation research forward into the clinical arena if, after being fully informed of the expected risks and benefits, patients voluntarily consent to it.

Half a century after Dr. Murray's epoch-making kidney transplant in 1954, innovative forms of transplantation continue to pose challenging ethical questions. We have, however, gained much assurance of the enormous value of transplants through 50 years of success. Yet at the beginning of this history, success was by no means guaranteed. At the beginning, there-

fore, we find courageous patients who, knowing how untried this procedure was, were willing to join in the novel venture with the surgeons and run the risks. Given the opportunity to choose, such patients, our study indicates, would also opt for facial transplantation.

Table 2. Risk acceptance of seven categories of transplantation by three samples*

	All Cases			Controls			Kidney transplant recipients			Facially disfigured		
	Mean	SD		Mean	SD		Mean	SD		Mean	SD	
Foot	21.131a	20.765		21.316	20.635		25.643	20.775		14.876	20.376	
Group	F = 2.552											
Difference	p = 0.08											
Hand	30.440b	20.692		29.790a	19.471		38.831b	21.054		23.057a	22.549	
Group	F = 5.849											
Difference	p = 0.003											
Larynx	37.870c	18.186		36.917	15.602		41.341	19.481		38.247	25.734	
Group	F = 0.998											
Difference	p = 0.370											
Kidney	42.574d	22.193		39.006a	23.561		53.202b	15.481		45.293a	18.572	
Group	F = 7.258											
Difference	p = 0.001											
Two hands	44.080d	18.391		45.420a	14.838		46.936a	20.443		34.803b	26.099	
Group	F = 5.420											
Difference	p = 0.005											
Hemiface	53.086e	23.572		54.013a	24.312		57.582a	17.819		43.638b	24.476	
Group	F = 3.667											
Difference	p = 0.027											
Face	59.203f	24.055		61.769a	23.689		59.577a	20.442		47.598b	26.796	
Group	F = 4.973											
Difference	p = 0.008											
Sample size, n	222			148			41			34		

*Means in the left column with different letter codes (a, b, c, and so on) or in the same row with different letter codes differ, $p < 0.05$.

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Chapter 14

The technical, immunological and ethical feasibility of face transplantation

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INTRODUCTION

The human face and facial transplantation has long captured the interest and imagination of scientists, the media and the lay public. This is not surprising since our faces are unique parts of our anatomy that like no other we associate with special qualities that make us uniquely human. Our face is much more than the anatomical location where our olfactory, auditory and visual organs are situated. We use facial expressions to communicate with the world around us and our face is the window through which others see and come to know us. We communicate these feelings in our spoken language with terms like "let's face it", "face to face", "maintain face" and "face value". It is this great importance we attach to our face that makes facial disfigurement such a devastating condition. Of all the physical handicaps, none is more socially devastating than facial disfigurement. In a large number of cases facial disfigurement leads to depression, social isolation, and even the risk of suicide^{1,2}. Rather than the sympathy or pity evoked by an amputated limb, a crutch, or a wheelchair, facial disfigurement elicits anxiety, fear and a wish to remove it from one's sight^{3,4}. In the words of a patient suffering with facial disfigurement: "I've spent fifteen years being treated for nothing other than looking different from everyone else. It was the pain from that, from feeling ugly, that I'd always viewed as the great tragedy in my life. The fact that I had cancer seemed minor in comparison"⁵.

Facial transplantation could provide an excellent alternative to current treatments for facial disfigurement caused by burns, trauma, cancer extirpation or congenital birth defects. As the introduction of solid organ transplantation provided an effective treatment for end stage organ failure and in doing so revolutionized the field of transplant immunology, so could facial transplantation revolutionize the field of reconstructive surgery for severe facial disfigurement. The introduction of organ transplantation into the clinical arena brought with it many technical, immunological and ethical issues that heretofore had never been seen by the scientific and lay communities. This is also the case with facial transplantation. The purpose of this editorial is to inform the reader of the major technical, immunological and ethical issues surrounding facial transplantation and to elicit professional discussion from the surgical community.

TECHNICAL ISSUES

Current methods of treating facial disfigurement consist of repairing or reattaching the original tissues, transferring autologous tissues from another part of the body, and using prosthetic materials to restore facial appearance and function. By far the best outcomes are achieved when the original tissues can be salvaged and used to reconstruct the defect. In cases when this is not possible either because the trauma or disease causing the loss destroyed the tissue beyond use (major crush injuries, severe burns, tumor invasion) or because the original tissues never existed in the first place (congenital birth defects) reconstructive surgeons must resort to autologous tissue transfers or prosthetic materials. In the former instance, skin grafts are used for simple wound coverage while skin and composite tissue flaps are used to reconstruct complex tissue defects. In the latter circumstance prosthetic materials are specially designed to camouflage the defect.

Over the past 20 years current treatment options have experienced many advances: *Skin grafting* has benefited from new grafting methods and new techniques used to care for the skin once it is transferred⁶. Techniques that enable the use of bioengineered skin products have greatly increased treatment options and improved outcomes⁷. *Skin and composite tissue flap transfer techniques* have revolutionized the field of reconstructive facial surgery. By enabling surgeons to reconnect very small blood vessels and nerves, advances in microsurgical techniques and instrumentation have made it possible to replant^{8,9} and transplant tissues from any part of the body to reconstruct complex facial tissue deficits^{9a,10,11,12,13}. *Prosthetic materials* are devices made of a variety of different synthetic materials. New materials have improved these devices with improved match (color and texture) to the tissues adjacent to the defect they cover^{14,15}.

In spite of these advances, current treatments for severe facial disfigurement are still far from ideal. While the methods that use autologous tissue do a good job of "filling in" the defect, the absence of facial tissues results in little to no functional recovery, the aesthetic outcomes are poor at best and the donor site from where the tissues are taken often present major problems. In some severely injured patients more than 100 procedures over periods of 10 to 20 years have been required. In these complex cases this extended series of reconstructions are fraught with complications, frequently fail to achieve the intended result, and often worsen the deformity. Treatments using prosthetic materials are excellent for giving the patient a normal static aesthetic appearance but they provide no functional or dynamic return, robbing the patient of his or her ability to communicate with facial expressions.

Facial transplantation: would make it possible to use healthy facial tissues (identical to the recipients' original tissues) to reconstruct the defect and thus provide better outcomes and eliminate many of the problems associated with current treatments. Facial transplantation would consist of removing facial tissues from a brain dead donor (solid organ donor) and transplanting it to a recipient to reconstruct the facial defect. The severely scarred and fibrotic tissue on the recipient's face would be removed and replaced with anatomically and functionally normal tissues, which over a period of 1-2 years would be expected to regain significant facial nerve function and animation.

Donor tissue procurement: When the donor tissue is located and confirmed to meet the pre-established inclusion criteria the recipient will be notified, brought to the hospital and prepared for surgery. At the same time members of the surgical team will accompany the solid organ procurement team to retrieve the donor tissue. The technical details of retrieving the donor facial tissues are challenging and technique dependent. It is expected that in most cases all of the soft tissue down to the bone will be needed to reconstruct a severely disfigured face. At its most basic, the donor facial tissue will be matched to and patterned from the defect defined by the recipient's deformity. This segment of tissue will include skin, subcutaneous tissue, muscle, and the arteries, veins and nerves necessary to satisfactorily perfuse and innervate the facial musculature of the transplanted facial tissue.

Facial tissue implantation: While surgical implantation will take many hours, the first surgical priority will be to revascularize the facial tissue retrieved from the donor so as to minimize the ischemia time. It is important to note that the tissues that will be transplanted in this procedure (skin, subcutaneous tissue, muscle) can withstand relatively long periods of ischemia¹⁶ therefore it is not anticipated that this will present a problem. If the defect requires that a full face be transferred, it is expected that four arteries and four veins would be reattached and as many as twenty facial motor nerve branches and major sensory nerves would be repaired. In the event the full face is not required to reconstruct the defect, proportionately fewer artery, vein and nerve repairs will be necessary. While reattaching multiple vessels to provide perfusion is the best case scenario, and will most likely be possible in facial transplantation procedures, it is well known that, due to its rich blood supply, face or scalp tissue can survive on only one good perfusing vessel¹⁷. Once the vessels are reattached and the

blood supply is restored to the transplanted tissue, the remainder of the reconstruction - reattaching the many delicate structures and nerves can be carried out in a methodical and unhurried fashion and could take as long as 8 to 16 hours to complete.

If it becomes necessary to remove the transplanted facial tissue, due to technical complications (thrombosis of the reattached vessels) or due to rejection (because the immunosuppressive drugs must be discontinued), either another donor would be identified for a second transplantation or the patient's treatment protocol would revert to conventional reconstructive methods (grafts, flaps, etc...) depending on the cause of failure.

In many respects current methods that repair and reattach damaged tissues or that remove, transfer and reconfigure autologous tissues to reconstruct facial deformities are more technically challenging than transplanting healthy facial tissues from a donor. The technical expertise and techniques needed to transplant human facial tissue are common practice and are performed daily in most centers where complex facial reconstructive procedures are performed. These methods have been developed and improved over the years and are the basis for current facial reconstructive and aesthetic techniques.

IMMUNOLOGICAL ISSUES

From an immunological standpoint, since the face and the hand contain mostly the same tissues it is reasonable to assume that the same immunosuppressive regimen found to be effective in human hand transplants should also work in face transplantation. In 1997 experiments in a large animal model¹⁸ demonstrated that a new immunosuppressive drug regimen widely used in organ transplantation (tacrolimus/MMF/prednisone) successfully prevented composite tissue allograft rejection, while causing minimal systemic toxicity^{19,20}. Based on these experiments in 1998 and 1999 teams in Lyon (France), Louisville (USA) and Guangzhou (China) performed the first 4 human hand transplants using this same drug regimen^{21,22,23}. The most common complications associated with the use of immunosuppressants include increased incidence of infections, malignancies, and end-organ toxicity. In the case of tacrolimus/MMF/prednisone combination therapy (the drug regimen that would most likely be used in facial transplantation), the incidences of these complications are as follows:

Infections: The incidence of opportunistic infections, including CMV, reported in kidney transplant recipients using tacrolimus and MMF range from 8.4% to 31%^{24,25}. When this complication occurs, the initial treatment usually consists of the appropriate antibiotic, antifungal, or antiviral agent. In rare cases it is necessary to lower the level of immunosuppression, or even to halt immunosuppression altogether.

Malignancies: In kidney transplant recipients (receiving similar doses of MMF at 2g/day as would facial transplant recipients) there exists a 1.2% incidence of post transplant lymphoproliferative disease (PTLD) and 11.1% incidence of non-melanoma skin carcinoma^{26,27,28}. In the case of heart, lung, or liver transplants, the only resources for treatment of these malignancies are surgery, irradiation, or chemotherapy. Due to the life-saving nature of these transplants, omission of immunosuppression would lead to rejection and consequently death. In the case of kidney transplantation, however, in addition to the appropriate oncologic treatment, immunosuppression is usually halted to restore the patient's immune responsiveness against the tumor. This would also be possible for facial transplantation, where the non-life saving nature of the transplant would allow the immunosuppressive treatment to be stopped without causing death.

End-organ toxicity: In solid organ recipients, tacrolimus has been reported to be associated with end-organ toxicity presenting itself in the form of post-transplant diabetes mellitus in 7 to 11.9%. Of these approximately 2/3 are able to discontinue insulin within 12 months after transplant²⁹. Tacrolimus is also nephrotoxic, as evidenced by increased blood creatinine levels in approximately 20% of the recipients using this drug. Since organ toxicity is relatively drug-specific, substitution with different drugs often offers a solution in these cases. Combining tacrolimus with MMF makes it possible to reduce the tacrolimus doses and thus diminishes nephrotoxicity while maintaining adequate immunosuppression.

It could be argued that in terms of immunosuppression-related end-organ toxicity, facial transplant recipients will be at an advantage over solid organ recipients. This advantage stems from the fact that by the time solid organ recipients receive their donor organ, they have often already experienced multiple organ problems from their underlying chronic disease. When they receive their transplanted organ, the immunosuppressive drugs they must take often further damage their already debilitated organs. In the case of facial transplant recipients, serious underlying chronic disease would exclude the patient from transplantation and therefore their organs

should be healthy. Therefore, it is reasonable to expect less end-organ toxicity from the immunosuppressive drugs in facial transplant recipients when compared with solid organ recipients. While it is not possible to predict long term rejection in facial transplantation one can draw some conclusions from preliminary findings in the more than 20 human hand transplants performed to date worldwide. With the exception of the first hand recipient (Lyon, France) who requested that his transplanted hand be removed (due to immunotherapy noncompliance and rejection) two years and four months posttransplant³⁰ all other cases have been reported to be successful. Functional and aesthetic recovery, for as long as 5 years post-transplantation, has been described as good and immunosuppressant related complications have been minimal. This success in animal research^{19,20} followed by the success of over 20 human hand transplants documents the feasibility of this concept and strongly suggests that from an immunological standpoint facial tissue transplantation would also be successful.

ETHICAL ISSUES

As in all medical advances there are many ethical issues surrounding facial transplantation. For a detailed list of these ethical issues the reader is directed to the recent publication produced by the Royal College of Surgeons; Facial transplantation; Working Party report³¹. It is beyond the scope of this editorial to address each individual ethical issue associated with facial transplantation. Instead, here we will discuss the risk vs. benefit equation associated with facial transplantation and provide a list of ethical guidelines our team is following as we move facial transplantation research into the clinical arena.

The risk vs. benefit equation: The question “do the benefits of facial transplantation justify the risks posed by the immunosuppressive drugs required to prevent rejection?” is at the center of the ethical issues surrounding facial transplantation. While the risks of immunosuppression are generally accepted for “life-saving” organ transplantation procedures, these same risks are questioned when it comes to “non-life saving” or “quality-of-life improving” procedures like face transplantation.

Risks: Since everyone’s understanding of risk is different, to assess the amount of risk different individuals are willing to accept to receive the ben-

efits of facial transplantation our team developed a questionnaire-based study to assess this situation³². Our initial findings from over 250 individuals in 4 populations (1. healthy normal subjects, 2. upper extremity amputees, 3. kidney transplant recipients and 4. individuals with facial disfigurements) questioned indicate that they would accept significantly more risk to receive a face transplant than a single hand, double hand, larynx, foot or even a kidney transplant³³. This latter point is interesting since kidney transplantation is a universally accepted treatment for which the risk vs. benefit ratio is largely unquestioned.

Benefits: Benefits associated with facial transplantation can be separated into functional, aesthetic and psychological. The relative importance of these three types of benefits is important when assessing risk vs. benefits in transplant candidates and will vary from patient to patient³⁴. For example a hand transplant provides predominantly functional and, to a lesser degree, aesthetic benefits. The combination of these 2 benefits contributes to the psychological benefit derived from this procedure. This was evidenced in Louisville's 1st hand transplant recipient, in his repeated statements that his transplanted hand gives him a sense of being 'whole' and 'complete'³⁵. In the case of facial transplantation the functional benefits would depend on the deformity and could include such gains as restoration of eye blink, chewing, swallowing, oral continence, speaking, facial sensation and facial expressions. The aesthetic benefits would improve the patient's body image and sense of self. While it could be argued which of these benefits would be more important, there would be no argument that both would have a major impact on the patient's psychological benefits.

Other considerations: The availability of donor tissue in facial transplantation will undoubtedly be a major challenge. In organ transplantation donor organ supply is the factor that limits the number of cases that can be performed^{36, 37}. In the case of facial transplantation the donor supply carries with it unique and important psychosocial and ethical issues. The relationship between facial appearance and one's identity³⁸ raises the question whether families will donate facial tissues if they believe that their deceased loved ones will be recognizable in the face of a recipient. To address this question, we are using several approaches. First, in the above mentioned survey we are asking individuals a series of questions that assess whether they think a face transplant recipient would look like the donor and if so, would they consider donating facial tissues of their loved ones (work in progress). Secondly, we are conducting human cadaver stud-

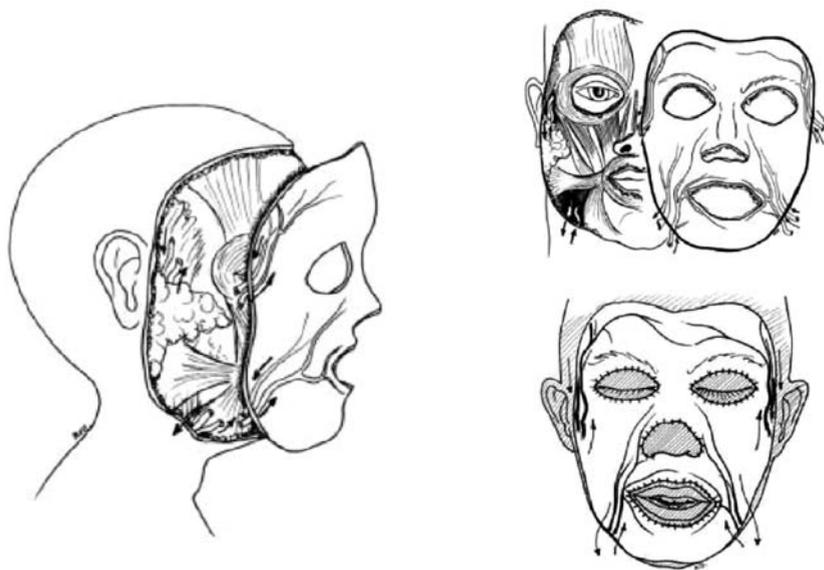
ies in which we transplant mask-like facial “soft tissues” from one cadaver to the bony skeleton of another and then assess whether the resulting soft tissue-bony skeleton combination can be identified by independent observers (work in progress). Initial findings from this latter study indicate that recipients do not look like the donor or the recipient but rather like a combination of the two. These preliminary findings are supported by others using computer generated imaging techniques³⁹.

To address the many ethical issues associated with facial transplantation, in 1997 our team adopted and has followed a set of ethical guidelines recommended in a 1988 publication by eminent surgeon and ethicist Dr Francis Moore⁴⁰ and a 1997 presentation⁴¹ and resulting publication⁴² by ethicist Dr Mark Siegler. Together these publications recommend that four criteria be fulfilled when introducing innovative surgical treatments: 1. Scientific Background of the Innovation; as many uncertainties as possible (see “equipoise” below) should have been clarified through well planned scientific research; 2. Field Strength; those who introduce a new surgical treatment should have the skill and experience to perform such a procedure; 3. Ethical Climate of the Institution; the motives of the institution should be centered on patient care and advancement of science; 4. Open Display and Public and Professional Discussion and Evaluation; this point is especially relevant to this editorial. At all stages of developing an innovative treatment there should be public and professional forums for open discussion and evaluation by peers and the general public. Examples of professional discussion are the present editorial, the Royal College of Surgeon’s Working Party report, 31 publications in scientific and clinical journals^{43,44,45,46} and presentations at scientific meetings^{47,48}. Forums for public discussion on facial transplantation have been in the form of public gatherings⁴⁹, website based discussions⁵⁰ and several forms of lay media. According to Moore and Siegler, if every effort has been made to follow these four criteria when introducing surgical innovations then clinical scientists may proceed.

The question of whether enough scientific research and preparation has been done to justify moving into the clinical arena is addressed by Siegler in his description of equipoise⁴². Equipoise refers to a situation of uncertainty in which the clinical investigator regards the potential outcome of a clinical trial as truly balanced between its potential for benefiting a patient and for causing unintended harm⁴². The key term here is “uncertainty.” At stake is an uncertainty that remains at the point at which one has gained as much knowledge as one can without actually performing the innovative

procedure. Therefore the only way to acquire the knowledge that is still lacking - the knowledge needed to resolve the uncertainty - is to actually carry out the innovative procedure in humans and see what happens. In the case of facial transplantation, particularly in psychological and societal issues, we find ourselves in a position of equipoise because we are destined to remain uncertain about whether the benefits will outweigh the harms (or vice versa) until we actually perform the procedure in humans and follow the outcomes.

In summary, we believe that for a select population of severely disfigured individuals facial transplantation, despite its recognized risks, could provide a better treatment option than current methods. The actual surgical techniques necessary to perform these procedures, while technically demanding, are commonly performed and are readily available today. From an immunological standpoint since face and hand contain mostly the same tissues it is reasonable to assume that the same immunosuppressive regimen found to be effective in human hand transplants should also work in face transplantation. While there are risks associated with these immunosuppressive drugs these risks have been extensively studied in large populations of solid organ transplant recipients and are well known and documented. The ethical issues associated with the risks and benefits of performing an innovative procedure of this type will always be present. To assure that facial transplantation moves into the clinical research phase in a thoughtful and well planned manner it is important that teams proposing to perform this procedure establish and follow well-defined ethical guidelines. The role of clinical scientists is to gather as much knowledge as possible about a new treatment from research, clinical experience, professional and public discussion and with this inform the patient and his/her family as best as is possible about the associated risks and benefits. As with all innovative medical advances ultimately it is the patient who must decide whether to be treated.



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Chapter 15

On the ethics of facial transplantation research

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INTRODUCTION: ADVANCES IN TRANSPLANT SURGERY AND ETHICAL CRITERIA

The field of transplantation surgery has always pushed the boundaries of medicine forward. In doing so it has repeatedly raised unprecedented ethical questions. Today, as teams around the world consider performing a human facial transplantation, the frontiers of medical ethics are again being tested. Not long ago the pressing ethical issues in transplantation concerned the scarcity of donated organs and the deaths of potential recipients that resulted from this lamentable scarcity¹. With the relatively recent advent of human hand transplantation, however, ethical reflection has shifted to the need to weigh the risks the patient assumes for the sake of receiving a donated organ that, unlike a heart or liver, is not necessary for his or her survival.

The aim of this essay is to address these ethical issues when they arise for human facial transplantation research. When considering facial transplantation research, the ethical concerns must be based on the scientific, surgical, psychological, and social dimensions of the procedure and its aftermath. Therefore, this article devotes considerable space to discussing these dimensions in so far as they have implications for ethics. The ethical questions that arise here are complex and, as we have indicated, unprecedented. Issues of the psychological hopes, anxieties, and stability of transplant recipients have always caused ethical concerns, but with facial transplantation the psychological and social dimensions loom much larger: what is at stake is a person's self-image, social acceptability, and sense of normalcy as he or she subjectively experiences them. To formulate these broad concerns in the language of medical research ethics, many of the "risks" and "benefits" of the surgery seem unpredictable.

As one of the teams preparing to perform human facial transplantation, a key part of our program at the University of Louisville consists of soliciting and incorporating professional discussion into our protocol. The purpose of this essay is to present our reflections on human facial transplantation research to the biomedical ethics community in order to solicit their responses. We view this essay as a component of the "open display and public and professional discussion" required for proceeding in an ethical manner toward the performance of an innovative surgical procedure. As the reader will see below, this is one of the four ethical criteria that Dr. Francis Moore stipulated for undertaking such procedures^{2,3}. Our team adopted these criteria and is adhering to them as part our program's ethical guidelines. Throughout this essay we shall refer to the steps our team at the University

of Louisville has taken to meet both Moore's criteria and the ethical standards applicable to all health care research.

In part I of this article, we sketch the surgical procedures that are presently utilized in treating facial disfigurements.

In part II, we presuppose the guidelines and regulations formulated by *The Nuremberg Code*, *the Declaration of Helsinki*, *The Belmont Report*, and various official documents that form the basis for the ethical evaluation of all health care research performed today, and we examine facial transplantation from this point of view. We accordingly address the permissibility of facial transplantation research in terms of risk/benefit assessment, informed consent, and privacy and confidentiality.

In part III, we address the criteria enunciated by Francis Moore for judging the acceptability of innovative surgery²⁻⁴. Since we believe that Moore's criteria prompt us to focus on issues not routinely included in the ethics of research, we also deem it important to examine facial transplantation in the light of these requirements.

In part IV, we raise the question, Is it time to perform a facial transplant? Based on parts II and III we summarize eight criteria that, we think, must be satisfied in order to answer this question in the affirmative. We then consider that we have satisfied these criteria at the University of Louisville and that therefore it is justifiable to move forward with performing an experimental facial transplant.

I. PRESENT-DAY PROCEDURES FOR TREATING FACIAL DISFIGUREMENTS

Facial disfigurement can result from trauma, extirpation of tumors, major burns, severe infections, or congenital birth defects. Patients with such disfigurements number in the thousands⁵. The most advanced treatments available today consist of reconstructing these defects by surgically reattaching the original tissues^{6,7}, transferring autologous tissues from another part of the body^{8,9}, and/or using prosthetic materials to replace the missing tissues¹⁰. By far the best outcomes are achieved with the first alternative, when the original tissues can be salvaged and used to reconstruct the defect. Unfortunately, in most cases the original tissue cannot be salvaged, either because the trauma or disease causing the loss destroyed it beyond use or because the original tissues never existed in the first place (as in congenital birth defects).

When, as in most cases, the original tissues are not available, autologous tissue and/or prosthetic materials are used to reconstruct large tissue defects of the face. In these situations, complications caused by prosthetic materials (e.g., infection or rejection) are common, donor site morbidity (at the location from which the autologous tissues are taken) is almost always present, and multiple "revision" operations and prolonged rehabilitation are usually required. Moreover, functional and aesthetic recovery is usually poor, and the resulting deformity almost always leads to major psychosocial morbidity. The latter in turn often prompts these patients to retire to a secluded environment, becoming social recluses^{11,12}.

A possible solution to the above scenario is to reconstruct these severe facial deformities with identical tissues transplanted from brain-dead human donors (Composite Tissue Allotransplantation), as is done in solid organ transplantation. Composite Tissue Allotransplantation (CTA) in the form of human hand transplantation has recently received a great deal of attention in scientific circles and in the lay media. In the more than twenty hand transplants performed to date, the fact that the tissues used (human hands from brain-dead donors) were identical in both form and function to those originally lost has resulted in excellent early (five years) functional and aesthetic outcomes.

If facial transplantation were available for clinical application in the above-cited example, one could envision a single operation to replace the burned facial tissues with healthy donor tissues identical to the tissues destroyed in the accident. Following surgery, there would be a few revision operations giving the patient a normal appearance and nearly normal function, allowing him or her to return to a normal life in a relatively short time.

In spite of these advantages that facial transplantation has over current reconstructive methods, the main disadvantage is that patients receiving facial tissues from a donor would, like solid organ recipients, have to take potentially toxic immunosuppressive drugs for life in order to prevent rejection. The risks posed by these drugs raises the central question concerning facial transplantation: Do the benefits of facial transplantation justify the risks posed by the immunosuppressive drugs?

II. OFFICIAL ETHICAL CODES FOR RESEARCH ON HUMAN SUBJECTS

Here we shall address three of the main requirements of the ethics of research using human subjects: (1) risk/benefit assessments, (2) informed consent, and (3) privacy and confidentiality.

Risk/benefit assessments: Ethical codes governing medical and surgical research require careful risk/benefit analyses.

The Declaration Of Helsinki, states: Every biomedical research project involving human subjects should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the subject or to others. Concern for the interests of the subject must always prevail over the interest of science and society¹³.

The Belmont Report clarifies the extent of risks and benefits that need to be considered:

Many kinds of possible harms and benefits need to be taken into account. There are, for example, risks of psychological, physical, legal, social and economic harm and the corresponding benefits¹³.

The extent of risks and benefits may go beyond the individual subject, according to *The Belmont Report*: "Risks and benefits of research may affect the individual subjects, the families of the individual subjects, and society at large (or special groups of subjects in society)"¹³.

Risk/benefit assessments must be carried out by three different parties. The individual subjects themselves must make such comparisons. The investigative team must make them. And the Institutional Review Board (IRB) reviewing the research proposal must perform them. Regarding the IRB's duties, the U.S. Department of Health, Education and Welfare's Institutional Guide, *On the Protection of Human Subjects*, states:

The committee should carefully weigh the known or foreseeable risks to be encountered by subjects, the probable benefits that may accrue to them, and the probable benefits to humanity that may result from the subject's participation in the project or activity. If it seems probable that participation will confer substantial benefits on the subjects, the committee may be justified in permitting them to accept commensurate or lesser risks¹³.

Risk/benefit assessment in facial transplantation: In the light of these codes we must seek to develop a clear understanding of the risks to which a patient treated with a facial transplant would be exposed in comparison with the possible benefits. The main risks are those related to the

surgical transplant procedure and the lifelong immunosuppression medications that patients would have to take in order to prevent the transplanted tissue from being rejected. The expected benefits primarily would be improvements in quality of life in the form of restored function and aesthetic appearance and the concomitant improvement in the recipient's body image and sense of self. These benefits would probably also increase the recipient's ease and ability in social interactions with other people. While using transplanted tissues to reconstruct facial deformities would significantly improve a patient's quality of life, in most cases these procedures would not be life-saving in the strict sense of the word. This situation stands in contrast to life-saving treatments, like heart and liver transplants, in which the risk/benefit ratio is more readily conceptualized.

Below we discuss the risks and the benefits of facial transplantation and apply them to the "risk and benefit" lessons learned in solid organ transplants and the recent hand transplants.

General Risks of Organ Transplantation Compared to Face Transplantation

Risks related to surgery: While facial transplantation is a complex procedure, it does not pose more risks than conventional reconstructive procedures in which the patient's own tissue is used to repair the defects. In a 1998 multicenter study, Dupont et al.¹⁴ estimated this mortality to be no higher than 0.0567%, which was a figure far higher than that reported in most studies. In addition, compared to conventional reconstructive procedures, facial transplant procedures would utilize tissues taken from a donor rather than from the patient's own body and would thus obviate the complications associated with donor site morbidity. Also, conventional reconstructive methods can require over 100 revision surgeries over many years whereas, if successful, facial transplantation would require only a few surgeries. Since each surgical procedure carries with it inherent risks, it could be argued that conventional reconstructive methods are associated with more risks than facial transplants.

Risks related to immunosuppression: The immunosuppression-related risks in facial transplantation are also expected to be the same as those experienced by the solid organ and hand transplant recipients, who receive the same drug regimens. The most common complications associated with the use of immunosuppressants include increased incidence of: (1) infections, (2) malignancies, and (3) end-organ toxicity. In rare instances

malignancies associated with immunosuppressive therapy can result in death. The incidences of these complications, in the particular case of tacrolimus and mycophenolate mofetil/prednisone combination therapy (the drug regimen that would most likely be used in facial transplants), are as follows:

Infections: The incidence of opportunistic infections (bacterial, fungal, and viral, including CMV) reported in kidney transplant recipients using tacrolimus and mycophenolate mofetil (MMF) range from 8.4% to 31%^{15,16}. When this complication occurs, the initial treatment usually consists of the appropriate antibiotic, antifungal, or antiviral agent. In rare cases it is necessary to lower the level of immunosuppression, or even to halt immunosuppressive drugs altogether.

Malignancies: In transplant recipients, there exists a 1.2% incidence of posttransplant lymphoproliferative disease (PTLD) and an 11.1% incidence of nonmelanoma skin carcinoma (reported over a three-year period of follow-up)¹⁷. When malignancies occur in heart, lung, or liver transplant patients, immunosuppression must be continued because of the life-saving nature of the transplanted organ.

However, in facial transplantation, as in kidney transplantation, immunosuppression could be halted so that the patient's immune responsiveness against the tumor might be strengthened. Here, the recipient's life would not be put at risk by discontinuation of immunosuppression even though the consequence would be loss of the transplanted tissue.

End-organ toxicity: In solid organ recipients, tacrolimus has been reported to be associated with end-organ toxicity and presents itself in the form of post-transplant diabetes mellitus in 7 to 11.9% of recipients. Of these, approximately two-thirds are able to discontinue insulin within twelve months after transplant^{18,19}. Tacrolimus is also nephrotoxic, as evidenced by increased blood creatinine levels in approximately 20% of the recipients using this drug. Since organ toxicity is relatively drug-specific, substitution with different drugs often offers a solution in these cases. Combining tacrolimus with MMF makes it possible to reduce the tacrolimus doses and thus diminishes nephrotoxicity while maintaining adequate immunosuppression²⁰. In the case of end-organ toxicity, it could be argued that recipients of transplanted facial tissues have an advantage over solid organ recipients. This is due to the fact that facial tissue recipients could be potentially less susceptible to immunosuppression-related end-organ toxicity than solid organ recipients. This stems from the fact that by the time solid organ recipients receive their donor organ, they have often already experienced multiple organ problems from their underlying chronic disease. Once they

receive their transplanted organ, the immunosuppressive drugs they must take often further damage their already debilitated organs. In the case of facial tissue recipients, serious underlying chronic disease would exclude the patient from transplantation, and consequently their organs should be healthy²¹. Therefore, it is reasonable to expect less end-organ toxicity with the immunosuppressive drugs in facial tissue recipients when compared with solid organ recipients.

Psychological risks: The psychological risks that facial transplant recipients will confront will be similar to those experienced by solid organ transplant recipients, for example, a desperation that creates unrealistic hopes, fears that his or her body will reject the transplant, guilt feelings about the death of the donor, difficulty conforming to the treatment regimen and its side-effects, and a sense of personal responsibility for the success of the procedure²².

Moreover, the recipient of a new face must deal with a new appearance, but to some extent this resembles the risk of receiving a new hand, which also reshapes one's sense of one's appearance. What is unique to facial transplantation, however, is that facial appearance is intimately and profoundly associated with one's sense of personal and social identity. Therefore, the recipient of a face must adapt to his or her own responses to this new "identity" as well as to other people's responses to it. It is expected that such adaptations will not occur once and for all; rather, they will repeatedly occur and undergo modifications over time. Moreover, it will be impossible for the recipient of a transplanted face to escape a bright public spotlight, and such publicity will be invasive and long-term. Such risks might be mitigated by careful patient selection, ongoing monitoring, and psychiatric intervention, as indicated.

Social risks: As in cases of solid organ and hand transplantation, the family of the recipient of a face will be responsible for care-giving and social and psychological support. The recipient and his or her family will also be subjected inevitably to intrusive publicity and media coverage. In addition to these risks to the family of the recipient, there are other risks that we might imagine affecting the larger society. For example, a successful facial transplant might be interpreted as conveying the message that a good quality of life cannot be achieved by people with disfiguring conditions. There also exists the possibility that the public may develop unrealistic expectations for the outcomes of such surgery, perhaps to the point of creating an inappropriate demand for its use in less worthy cases, such as cosmetic

enhancement for the aging rich or for criminal identity concealment. The facial transplant research team cannot prevent these or other misconceptions. What the team can do is provide accurate information in order, it is hoped, to shape public opinions in a responsible manner.

General Benefits of Organ Transplantation Compared to Face Transplantation

Benefits associated with facial transplantation can be separated into three categories: functional benefits, aesthetic/psychological benefits, and social benefits. The relative value of these three types of benefits is important when assessing the risk/benefit equation for a transplant candidate and developing a triage strategy. For example, a hand transplant provides predominantly functional and, to a lesser degree, aesthetic benefits. The combination of these two benefits contributes to the psychological benefit derived from this procedure. A transplanted hand takes the place of the lost/missing hand in the spatial resolution of the patient. This has important psychological implications and is a great benefit of this procedure. This was clear in the repeated statements by Louisville's first hand transplant recipient, in which he asserted that his transplanted hand gave him a sense of being "whole" and "complete"²³.

Functional benefits: Functional recovery of the facial tissues offers several important benefits. Depending on the extent of the original deformity, the anticipated benefits include restoration of blinking for eye protection, improved oral continence, and restoration of facial expression and sensory function.

Aesthetic and psychological benefits: "The human face is unquestionably the most important aesthetic anatomical feature of the human body. Much of how other people react to us depends upon our aesthetic appearance. Moreover, the appearance of our face is the predominant anatomical feature by which we identify and differentiate ourselves from others. In a large number of cases facial disfigurement leads to depression, social isolation, and even the risk of suicide^{24,25}. By replacing the disfigured face with a "normal" appearing/functioning face, facial transplantation would provide important psychological benefits.

Social benefits: Closely related to functional, aesthetic, and psychological benefits is the enhanced social capacity of the subject. Although a pe-

riod of adaptation will be required for both the subject and others involved, the subject's willingness and ease in engaging in social interactions should improve. Restoring the abilities to make facial expressions, enjoy an aesthetically acceptable appearance, and interact comfortably with others lends significant weight to the benefit side of the risk/benefit equation.

Informed consent: Ever since *The Nuremberg Code*¹³, informed consent has been fundamental to any research performed with human subjects. *The Belmont Report* grounds this requirement in the basic ethical principle of respect for persons¹³. The *Report* states:

Respect for persons requires that subjects, to the degree that they are capable, be given the opportunity to choose what shall or shall not happen to them. This opportunity is provided when adequate standards for informed consent are satisfied¹³.

On the Protection of Human Subjects: U.S. Department of Health, Education and Welfare's Institutional Guide specifies the main items to be covered by informed consent:

The basic elements of informed consent are: A fair explanation of the procedures to be followed, including an identification of those which are experimental; A description of the attendant discomforts and risks; A description of the benefits to be expected; A disclosure of appropriate alternative procedures that would be advantageous for the subject; An offer to answer any inquiries concerning the procedures; An instruction that the subject is free to withdraw his consent and to discontinue participation in the project or activity at any time¹³.

All prospective candidates being considered for facial transplantation in our program will be presented with an informed consent in both oral and written form. Investigators who will be involved in performing the transplant will discuss with the prospective subject all elements of the informed consent and will address any concerns or questions that the subject may have. Prospective subjects will in no way be coerced or manipulated regarding any part of the informed consent process.

To assure that the prospective candidate receives an objective perspective during and after the informed consent process, he or she will be encouraged to select a subject advocate who will assist him or her in understanding and deliberating about the various components of the procedure.

Here we shall not summarize the many items included in this informed consent process. However, we would like to note that item 6 in the *On the Protection of Human Subjects: U.S. Department of Health, Education and Welfare's Institutional Guide* cited above cannot be followed strictly in fa-

cial transplantation: the subject must conform to the research-treatment regimen as long as he or she has the transplanted facial tissue.

Privacy and confidentiality: *The Declaration of Helsinki* states: The right of the research subject to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the subject and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject¹³.

In the case of transplantation research, there are two groups of persons whose privacy and confidentiality should be respected. The first is the donor and his or her family, and the second is the recipient and his or her family.

Facial tissue donor: The privacy and confidentiality of the donor and his or her family ought to be respected to the extent permitted by law. All reasonable efforts should be made to protect the donor's anonymity. Identifying information ought not to be publicly revealed. The donor's family must be informed, however, that the research team cannot prevent someone (e.g., a member or friend of the donor's family) who knows about the case from publicizing information on his or her own.

Facial tissue recipient: In the case of an innovative therapeutic procedure like facial transplantation, there are two reasons for concern about the confidentiality and privacy of the recipient and his or her family:

The full scientific reporting and discussion of this procedure and its results may be restricted too greatly by efforts to maintain the privacy of the subject. For example, in the publication of the outcomes of the operation it may be highly desirable, from a scientific point of view, to provide unaltered photographs of the face of the recipient. Also, in conference presentations it might be very helpful, again from a scientific point of view, to hear the recipient him- or herself speak about his or her experience and to respond to questions. Hence the mandate to respect privacy and confidentiality may conflict with scientific requirements.

The prospect of a facial transplant has already attracted significant media attention, and as the likelihood –and then the reality– of such a phenomenon develops, the interest of the media in it will inevitably become greater. It is difficult to imagine, then, how the media can be kept from discovering the identity and much other information about the recipient and his or her family. Indeed, for the recipient, “privacy” may not be possible.

In a recent article entitled "High-Profile Research and the Media: The Case of the AbioCor Artificial Heart," E.H. Morreim carefully examined the issue of disclosure of information to the public²⁶. She pointed out that, from a scientific point of view, the ideal way to provide information to the public is through publications in refereed professional journals. Peer-reviewed publications are better able to provide accurate scientific information than are press releases that occur as the research project progresses. Nonetheless, she noted that high-profile research cannot enjoy such luxury in a society that prides itself on its "freedom of the press." She sought, then, to sort out the competing obligations to disclose information to the public, to maintain the research subject's privacy and confidentiality, and to publish the procedures and results of medical/surgical research in professional journals. Morreim²⁶ pointed out that in our society we must recognize "the right of free press" and the public's "desire to know" about health care innovations. Freedom of the press, she asserted, "does not mean that anyone is required, in the first place, to provide a reporter with whatever information he wants"²⁶. Similarly with the public's "desire to know" some kind of information: it does not entail that anyone has the duty to produce the information²⁶.

Nevertheless, in keeping with our established policy of "open display and professional and public discussion and evaluation," we believe we are obligated to release to the press basic clinical and surgical information about facial transplants. This obligation, however, must be balanced against the research subject's right to privacy and confidentiality. Morreim seems to have concluded that "materially significant trends in the progress of the trial" should be disclosed to the public, and "Patients should not be permitted to veto the disclosure of such information"²⁶. Affirming the patient's right to privacy, however, she added,

Patients should be able to control some kinds of information. Clearly, purely personal details such as marital status, education, occupation, and the like should be governed by the patient²⁶.

And, she continued, Additionally, patients and families should have the opportunity to review press releases in advance to correct errors, delete unsuitable personal information, and influence the tone of the report²⁶.

These suggestions will guide our approach to press releases and to protecting the subject's privacy and confidentiality. Accordingly, we shall inform the subject and his or her family that we shall need to publish in professional journals some identifying information about the subject. We shall seek, however, to restrict such information to solely what is necessary for scientific purposes. In addition, we shall inform the subject at the outset that

we shall provide press releases. As press releases are prepared, the general nature of the information that will be released will be disclosed to the subject and the subject's family, and they will be given the opportunity to review the information and offer suggestions. We shall also inform the subject that extensive media attention is likely to be forthcoming and that we cannot guarantee that their identities and other personal information will not be discovered and published by the media. As the press releases are prepared, we shall provide subjects and their families the opportunity to review them in advance and offer suggestions. Subjects and their families will remain at liberty to control personal information in so far as this can be done in the light of the intense media spotlight.

III. FRANCIS MOORE'S CRITERIA FOR INNOVATIVE SURGICAL PROCEDURES

In our facial transplantation program at the University of Louisville, in addition to the above ethical requirements, we have also adopted and are following criteria recommended by Dr. Francis Moore (1988, 1989). In 1988 article, Moore offered four criteria for determining whether it is ethically acceptable to employ an innovative surgical technique. His criteria were: (1) the scientific background of the innovation, (2) the skill and experience of the team ("field strength"), (3) the ethical climate of the institution, and (4) open display and public and professional discussion and evaluation.

The scientific background of the innovation: This criterion requires that the scientific preparation for proceeding to carry out an innovative surgical procedure must have been carefully and fully developed. The scientific preparation for facial transplantation is derived primarily from solid organ and hand transplantation research. In addition, unique to hand and facial transplantation, the risk vs. benefit equation in these non-life-saving procedures is being studied²⁷.

The vast majority of solid organ transplantation research that bears relevance to facial transplantation has focused on identifying and developing new immunosuppressive drugs and drug combinations that effectively suppress rejection while also causing minimal side effects. The relevant literature is full of basic science and clinical research describing the development and evaluation of these drugs²⁸. In 1997, experiments conducted in our laboratory in a large animal model²⁹ demonstrated that one of these new drug combinations (tacrolimus/MMF/prednisone) successfully prevent-

ed rejection of transplanted skin, muscle, bone, and other tissues making up the hand while causing minimal systemic toxicity^{30,31}. Based on these experiments, teams in Lyon (France), Louisville (USA), and Guangzhou (China) performed in 1998 and 1999 the first four human hand transplants using this same drug regimen³²⁻³⁴.

From an immunological standpoint, since the face contains mostly the same tissues as the hand, it is reasonable to assume that the same immunosuppressive drug regimen found to be effective in the animal research that preceded human hand transplants and in the human hand transplants that followed should also be effective in facial transplantation.

In addition to this animal research, the scientific preparation for facial transplantation must include empirical studies that address the critical ethical questions that such procedures pose. We are therefore in the process of carrying out several studies that aim to answer the central question, "Do the benefits of facial transplantation justify the risks posed by the immunosuppressive drugs required to prevent rejection?" While the risks of immunosuppression are generally accepted for "life-saving" organ transplantation procedures, these same risks are questioned when it comes to "non-life-saving" or "quality-of-life improving" procedures like facial transplantation. To address this issue we designed a questionnaire-based study²⁷ to assess the amount of risk individuals are willing to accept to receive the benefits of facial transplantation. Our initial findings from over 250 individuals in four populations questioned (healthy normal subjects, upper extremity amputees, organ transplant recipients, and individuals with facial disfigurements) indicate that they would accept significantly more risk to receive a facial transplant than a single hand, double hand, larynx, foot, or even a kidney transplant³⁵. The last point is intriguing since kidney transplantation is a universally accepted treatment for which the risk vs. benefit ratio goes largely unquestioned.

Siegler has claimed that central to the ethical concerns with respect to these procedures is the question of whether "the equipoise consideration has been satisfied." He defined equipoise as "a situation of uncertainty in which the clinical investigator regards the potential outcome of an experiment or clinical trial as truly balanced between its potential for benefiting the patient or for causing unintended harms"⁴. The key term here is "uncertainty." At stake is an uncertainty that remains at the point at which we have gained as much knowledge as we can through scientific studies; and therefore, additional knowledge can be attained only by actually performing the experimental procedure and following the outcome. We believe that facial transplantation has reached a position of equipoise because we are destined to

remain uncertain about whether the benefits will outweigh the harms (or vice versa) until we perform the procedure and observe the actual results.

The skill and experience of the team (“field strength”): Moore^{2,3} emphasized that the skill and experience of the team undertaking the innovative procedure is crucial. Obviously, such a procedure can be truly “tested” for its safety and efficacy only if the skills and experience of the team performing the procedure are unlikely to be the cause of failure. Moreover, the “field strength” of the team must be assured in order to protect the subjects from harm. *The Nuremberg Code* enunciates this ethical concern for beneficence: ⁸. The experiment should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment¹³.

The team at the University of Louisville is composed of experts who have extensive experience in the scientific, clinical, surgical, and psychological areas pertinent to facial transplantation. This includes specialists in reconstructive surgery, head and neck surgery, transplant surgery, immunology, psychology, psychiatry, ethics, Institutional Review Board participation, and organ procurement. The reconstructive and head and neck surgeons on our team are familiar with and regularly employ the latest techniques described above to remove, transfer, and reconfigure autologous tissues to reconstruct facial deformities. Indeed, members of the team have pioneered many of the techniques used today for reconstructing complex facial deformities³⁶. In addition, the team has acquired relevant skills and experience through having established a program for and performed successful human hand transplants. It is such “field strength,” we think, that is necessary in order to take the next step of performing a human facial transplantation.

Ethical climate of the institution: What is at stake here is ultimately the motivation for undertaking the innovative procedure. Moore was concerned that the innovation not be performed mainly for the purposes of institutional or professional self-aggrandizement. He thought that it should rather be carried out primarily for its potential contributions to those people who are in need of the procedure. As he expressed it:

When the epiphenomena of medical care, such as capital gain, investor profit, institutional representation, surgeon ego, municipal pride, and chauvinism, become the true objective of the procedure, then the ethical climate of the institution is no longer acceptable for therapeutic innovation^{2,4}.

Adherence to this ethical requirement is essential but difficult to verify. How can we determine what a person's or an institution's motivations are? Usually people and institutions engage in sizable projects with a variety of motives for doing so.

We suggest that the ethical issues here pertain to possible conflicts of interest. If desires for enhanced reputation, financial reward, professional vanity, and so on motivate those involved to compromise the scientific, medical, surgical, or ethical aspects of the procedure, "then the ethical climate of the institution is no longer acceptable for therapeutic innovation." An institution may seek an enhanced reputation and even financial profit from being "the first" to advance therapeutic techniques. Indeed, numerous health care institutions highly prize their public reputations for being "first" with innovative procedures, and this usually does not lead people to suspect unethical conduct. The desire to be first becomes unethical only when it motivates the institution to undertake the innovation in a manner that fails to follow strict scientific, medical, surgical, and ethical demands. The key question then becomes this: Have the institutions and professionals involved adhered as much as can reasonably be expected to scientific, medical, surgical, and ethical requirements in performing this new procedure? If these requirements have been met, then it matters little what other motivations may be operative. And this would seem to be the case especially in view of the fact that such motivations can usually not be detected or proven.

Open display and public and professional discussion and evaluation: Moore³ recognized that it is crucial that innovative surgical procedures be openly displayed before the broad community of professionals in the field as well as before the general public. In order to ensure that the issues surrounding facial transplantation would be submitted to public and professional discussion, evaluation, and criticism, we at the University of Louisville have organized and participated in several conferences addressing these manifold issues. Moreover, we have published the proceedings from these conferences in trade journals to make them accessible to as wide a professional audience as possible. Feedback we have received from public and professional discussion has allowed us to rethink and revise various components of our program. In fact, although our institutional review board proposal has been virtually complete for over three years, we have postponed submitting it for approval and have rather repeatedly fine-tuned it based on criticisms we have received from professional and public discussions.

Below we list the main examples of efforts we have made to meet Moore's recommendation of open display and public and professional discussion and evaluation.

In November 1997, we hosted the first International Symposium on Composite Tissue Allotransplantation in Louisville, Kentucky. The workshop brought together international experts in immunology, transplant, plastic, and hand surgery, research, and ethics to evaluate the scientific, ethical, and clinical barriers standing in the way of performing the first human hand transplants. After two days of discussion the consensus was reached that sufficient animal research had been done and that it was time to move on to the clinical phase of this research³⁷.

In May 2000, we convened the 2nd International Symposium on Composite Tissue Allotransplantation in Louisville, Kentucky to share the early results of the first human hand transplants and invited teams who had performed other types of composite tissue allotransplantation procedures (namely, larynx, bone, tendon, and nerve). Three hand transplant teams reported encouraging early immunological and functional findings. They reported that the immunosuppressive drug regimen [tacrolimus/MMF/Prednisone] they were using effectively prevented hand rejection, allowed for good recovery of hand function, and caused minimal toxic side-effects in their first patients³⁸.

We have also published discussions of the present and future state of composite tissue allotransplantation in professional trade journals³⁹⁻⁴¹.

On November 19, 2003, our team participated in a public discussion at the Dana Center of the London Science Museum. At this gathering four professionals from various fields related to facial transplantation explained their work and their respective positions on the question of whether the time had come to perform human facial transplants. This two-hour event specifically focused on the public's participation and their opinions⁴². Following this meeting the proceedings were posted on the Dana Center's website, and the public was invited to post its views. Finally, in addition to these public forums for discussion, we have also openly made our program available to the public in several sources of print, radio, and television media.

IV. IS IT TIME TO PERFORM A FACIAL TRANSPLANT?

In light of the above discussion we would like to put forward a set of criteria for determining whether the point has been reached, in the preparation and development of this innovative surgical procedure, at which it is justi-

fied to perform an experimental facial transplant. The criteria we propose are these:

Moore's criterion of "scientific background of the innovation." The preparatory scientific groundwork has been laid through laboratory and clinical investigations of the pertinent medications, technology, procedures, and ethical issues. This preparatory work has significantly reduced the risks of the proposed procedure.

Moore's criterion of "skill and experience of the team ("field strength")." The surgeons and clinicians involved in the research project possess the knowledge, experience, skills, and technical abilities needed for it.

Moore's criterion of "open display and public and professional discussion and evaluation." Items (1) and (2) above have been publicized so that professional and lay persons who have so wished have had sufficient opportunity to discuss and criticize the performance of the procedure. Moreover, these responses and criticisms have been seriously considered by the research team and have, when appropriate, influenced the revision of the research proposal.

Moore's critique of the "ethical climate of the institution." The innovation is not being performed for purposes of institutional prestige or professional recognition. It is rather the criteria enumerated here that are the truly governing ones.

The remaining uncertainties regarding facial transplantation and its consequences can be resolved either by proceeding to actually performing the procedure on human subjects or by postponing it and waiting for further developments. Undoubtedly, postponing the procedure would allow for the development of medical innovations. An analogy can be imagined in the manned mission to the moon. This venture would have been aided by the development of the microcomputer, digital camera, and other innovations produced during the past three decades. Such innovations, however, were not essential for a successful moon mission. We submit that, in an analogous way, future medical developments will provide only minimal knowledge compared to that which will be gained from performing the procedure. An example of this is in the knowledge gained from performing human hand transplants. Despite the arguments made against them as too precipitous and uncertain, over twenty hand transplants have been performed. As a result, the field has gained a wealth of knowledge based on direct evidence that would not have been possible if we had not dared to perform the procedure in the face of the uncertainties.

There exist informed subjects who, deeming the procedure beneficial, want to undergo it and who will not be able to undergo it if it is postponed in order to wait for further developments.

There exist indefinitely many other potential subjects who could in the future benefit from this procedure if it proves to be successful.

The procedure has been subjected to the established regulatory scrutiny and reviews, including approval by the relevant IRB.

If these eight criteria are satisfied, we submit that it would be justified to actually perform the experimental procedure on qualified, voluntary, and informed human subjects. Furthermore, we maintain that at the University of Louisville these criteria have been satisfied for the procedure of human facial transplantation. There arrives a point in time when the procedure should simply be done. We submit that that time is now.

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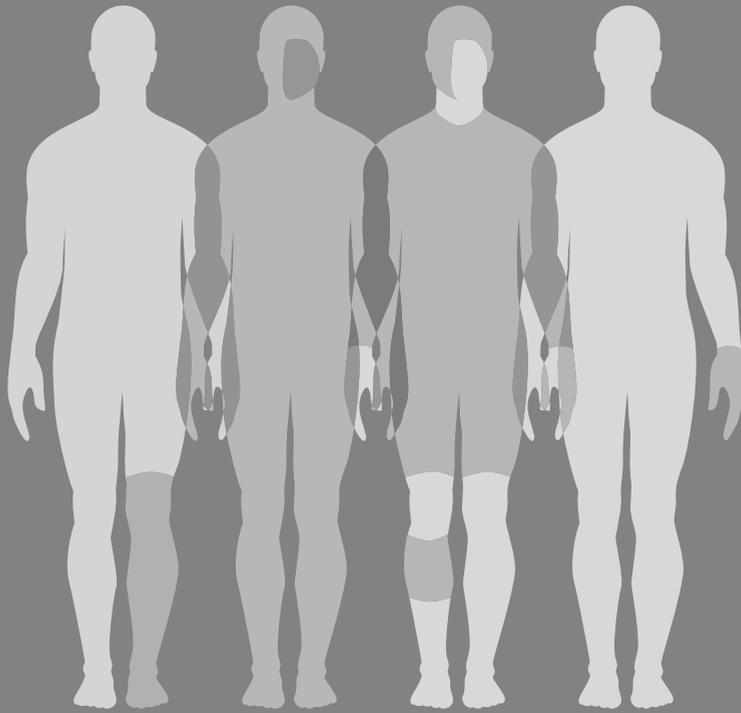
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Part IV





Summary and Conclusions

Each year in the USA alone, an estimated 7 million people need composite tissues to reconstruct large tissue defects resulting from trauma, extirpation of tumors, major burns and congenital malformations. Composite tissue allotransplantation (CTA) in the form of hand and facial tissue transplantation are now a clinical reality. While CTA is a promising new treatment option for reconstructing large tissue defects, the need for immunosuppressive agents to prevent rejection in these procedures poses a major problem. Even the most modern immunosuppressive drugs used today bring with them significant risk, including an increased incidence of neoplasm, opportunistic infections, and end organ toxicity such as drug induced bone loss. Therefore in **PART I** we looked at different combinations of immunosuppressive regimens in both a porcine and rat model. In particular the effect of the immunosuppressive regimens on bone quality was investigated.

A possible alternative to using chronic non-specific immunosuppression is to create a stable state of tolerance to the transplanted tissue. One way to induce such tolerance is to create chimerism. In **PART II** chimerism is studied as a way to induce tolerance in CTA recipients. Especially the role of graft-versus-host disease is studied in chimeric recipients of composite tissue allografts.

In **PART III** we aim at the future and look at risk acceptance and ethics in CTA. Since the introduction of facial transplantation in the clinical arena the ethical debate on risk versus benefit in CTA procedures, especially facial transplantation, has become even more meaningful.

PART I

In **chapter 1** a Cyclosporine A (CsA) based combination immunosuppressive regimen was studied in a porcine composite tissue allotransplantation model. This regimen of CsA, Mycophenolate Mofetil (MMF) and prednisone was at the time commonly used in vascularised bone and joint allotransplantation. The purpose of this study was to determine what effect CsA-based combination therapy has on bone quality and healing. Ten pigs received vascularized bone allografts with skin and muscle components (osteomyocutaneous free flaps) from size-matched donor animals. Recipient animals received oral CsA/MMF/prednisone therapy for 90 days. Bone quality was studied pre- and post-transplant by measuring the bone's acoustic velocity and density and by calculating the bone's elastic coefficient. Bone healing was assessed using radiographic analysis. We found that four animals were lost due to graft-rejection or immunosuppression related

complications before the 90-day endpoint of the study. While bone specimens taken from the 6 animals that completed the 90-day protocol had histological signs of rejection, they all appeared to have normal bone healing. Bone density values were significantly decreased post-transplant as compared to pre-transplant values. Results of the acoustic velocity and elastic coefficients measurements showed a significant decrease in post-transplant values, indicating diminished bone quality. Our findings indicate that CsA/MMF/prednisone combination therapy is ineffective in preventing bone rejection, decreases bone quality and it is associated with systemic toxicity suggesting that this immunosuppressive regimen at the doses used in this study is not ideal for CTA procedures such as vascularized bone allotransplantation.

Since the patients that received human hand transplants all used Tacrolimus (FK506), MMF and prednisone immunosuppressive therapy, this regimen was investigated in **chapter 2**. FK506/MMF/prednisone combination immunosuppression therapy has been found to effectively prevent composite tissue allograft rejection with minimal toxicity in a preclinical porcine model. These findings have been reproduced in 24 human hand transplant procedures in 18 patients. In CTAs containing bone, adequate bone quality and healing are essential for long-term functional success. The purpose of the study presented in this chapter was to determine the effect FK506/MMF/prednisone immunotherapy has on bone quality and healing. Forelimb CTA-flaps were transplanted in nine pigs. Recipient animals received FK506/MMF/prednisone therapy for 3 months. Bone quality was studied pre- and post-transplant by measuring acoustic velocity and density and by calculating elastic coefficients. Additional bone quality analyses were performed on unoperated limbs, and in bone grafts from two pigs that had autograft procedures performed. Bone healing was assessed using radiographic analysis. Three animals were lost to immunosuppression related complications before the endpoint of the study. The bone component of all six CTA-flaps showed normal healing. Although results of the bone density measurements were not significantly different when comparing pre- to posttransplant values, acoustic velocity and elastic coefficient measurements showed a significant decrease posttransplant indicating a decrease in bone quality. The transplant procedure itself appeared to decrease bone quality more than the immunosuppression regimen did over the observation period in this study. FK506/MMF/prednisone combination therapy prevented rejection and showed normal bone healing. Based on these findings, we conclude to prevent CTA failure it is important to monitor bone quality posttransplant.

Corticosteroids are known to have a detrimental influence on bone. However, in the clinical situation of allotransplant patients using combination immunosuppressive regimens, it is difficult to distinguish between the effects of the calcineurin inhibitors, such as FK506, and those of corticosteroids. Therefore in **chapter 3** we describe a study to determine whether a low dose, corticosteroid-free combination regimen of FK506 and MMF would prevent rejection in a rat composite tissue allotransplant model with minimal toxic side effects. Three groups were used in this study. In group I, Wistar Furth (WF) rat recipients received hind limbs from syngeneic WF donors. In groups II and III, WF rats received a hind limb from allogeneic August X Copenhagen Irish (ACI) rat donors. Rats in group III were treated with FK506-MMF for 5 months. Assessment for rejection, flow cytometry and mixed lymphocyte reactions were performed. Biopsies were taken regularly and at the time of sacrifice. Combination therapy with low dose tacrolimus-MMF effectively prolonged CTA survival indefinitely, with minimal side effects. Toxicity associated with immunosuppressive drugs can be avoided in a low dose combination corticosteroid-free regimen.

Therefore, in **chapter 4**, bone quality and healing is studied in a rat hindlimb allotransplantation model using this low dose combination corticosteroid-free regimen to prevent rejection. Six groups of tibiae were studied. Group 1 consisted of WF tibiae from the right hind limb that was removed at the time of transplant (WF pre-transplant). Also at that time the tibia from the non-transplanted ACI left hind limb was harvested (group 4: ACI pre-transplant). At the time of sacrifice non-transplanted WF left hind limbs (group 2: WF post-transplant) and transplanted ACI right hind limbs (group 5: ACI post-transplant) were removed and the tibia bones were used for measurements. Note that the hind limbs in group 2 were not transplanted, but the tibiae had been exposed to 5 months of systemic immunosuppression. At the end of the study tibiae were harvested from naive WF and ACI rats. These served as control group 3 (control WF) and 6 (control ACI) respectively. Bone quality was studied pre- and post-transplant by measuring acoustic velocity and density and by calculating elastic coefficients. We demonstrated that the acoustic velocity and the elastic coefficient of transplanted allogeneic and non-transplanted autologous bone were significantly lower when compared with age-matched non-transplanted, non-immunosuppressed control animals. Since this effect occurs in both the allogeneic transplanted and the autologous non-transplanted bone, it is most likely caused by the immunosuppressive regimen used in our study. Therefore, to maintain bone quality in composite tissue allotransplants, new treatment modalities or new immunosuppressive regimens need to be evaluated.

PART II

Despite improved immunosuppressive regimes, effective prevention of chronic graft rejection is not achieved and long-term survival of all grafts is limited. Chronic rejection is the major cause of late allograft loss. Even with the use of modern immunosuppression, approximately 35% of heart, liver and cadaveric renal allografts are lost within five years. Five-year survival of lung grafts is 42% and 35% for pancreas grafts. The induction of transplantation tolerance is one of the major goals in transplantation immunology. One of the most effective and best-studied approaches to achieve this goal is through bone marrow transplantation (BMT), which results in hematopoietic stem cell chimerism. However, the induction of chimerism by BMT has its own risks, which must be reduced or eliminated for this procedure to become a clinical reality. **Chapter 5** reviews and discusses the use of BMT for induction of chimerism and donor-specific tolerance with special emphasis on approaches to overcome the current limitations.

Transplantation of unmanipulated donor specific limbs to chimeric recipients results in severe graft-versus-host-disease (GVHD). This suggests that non-tolerant mature donor-derived cells in the CTA may affect the stability of chimerism, potentially resulting in GVHD. The aim of the study in **chapter 6** was to develop an approach to study and prevent GVHD in a mixed-chimeric-rat hind-limb transplantation model. In this study [ACI→WF] chimeras received a limb from: WF (syngeneic), Fisher (third-party) or ACI (irradiated [1050 cGy] or non-irradiated) rats. In vitro tolerance was assessed using mixed lymphocyte reactivity assays at the time of euthanasia. We demonstrated that [ACI→WF] chimeras with >85% chimerism exhibited rejection-free survival of donor specific hind limbs. However, 100% of these animals developed lethal GVHD, 22.4±2.8 days after limb transplantation. [ACI→WF] Chimeras transplanted with irradiated ACI or syngeneic WF limbs showed no signs of rejection or GVHD at five months. Non-chimeric and third-party controls rejected limbs within 10 days. We concluded that conditioning of host WF rats with 950 cGy of irradiation led to high levels of chimerism without GVHD. The mature T-cell content of non-irradiated donor limbs was sufficient to induce lethal GVHD in 100% of animals. Irradiation of donor limbs resulted in long-term donor tolerance and prevented GVHD. These data demonstrate that: 1) established chimeras could be susceptible to GVHD caused by immunocompetent donor cells transferred with the hind limb, and 2) that inactivating these cells with irradiation prevents GVHD, destabilization of chimerism, and permits rejection free graft acceptance.

We and others have shown that mixed allogeneic chimerism induces donor-specific tolerance to composite tissue allografts across major histocompatibility complex barriers without the need for immunosuppression. However, a delay period between bone marrow transplantation and limb allotransplantation is required, making such protocols impractical for clinical application. The study in **chapter 7** eliminates this delay period in a rat hind limb allotransplantation model by performing mixed allogeneic chimerism induction and transplantation "simultaneously." We studied a total of four groups. Group 1 included controls in which naïve WF hosts received ACI hind limbs. Group 2 included [ACI→WF] chimeras that received limbs from third-party donors (Fisher), and group 3 included chimeras that received irradiated (1050 cGy) ACI limbs. In group 4, WF hosts conditioned with 950 cGy received irradiated (1050 cGy) ACI limbs followed by infusion of 100×10^6 ACI T-cell-depleted bone marrow cells and immunotherapy (tacrolimus and mycophenolate mofetil) for 28 days. Group 5 animals received the same treatment as group 4 animals without immunotherapy. The rats in groups 1 and 2 rejected their limbs within 10 days. Only one rat in group 4 survived to the end of the study. Groups 3 and 5 demonstrated long-term limb survival without rejection or graft-versus-host disease. High levels of donor chimerism (>80%) were achieved and maintained throughout the study. Mixed lymphocyte reaction assays in both groups revealed donor-specific hyporesponsiveness with vigorous third-party reactivity. This study demonstrated that infusion of donor bone marrow cells into conditioned hosts immediately after limb transplantation results in stable mixed chimerism, robust tolerance, and reliable limb allograft survival.

After we demonstrated that in experimental tolerance protocols using mixed allogeneic chimeras, rat hind limb transplantation caused lethal GVHD in mixed chimeric hosts, we designed a study (**chapter 8**) to determine whether the lymphocytes within the bone marrow (BM) and/or lymph nodes (LNs) transplanted with the limb cause GVHD. We studied three groups. [ACI→WF] chimeric rats received ACI hind limbs that were non-irradiated, irradiated (1050 cGy) or had all LNs surgically excised. Rejection, GVHD and donor chimerism was assessed and mixed lymphocyte reaction assays were performed at sacrifice. The amount of $\alpha\beta$ -TCR⁺ T cells within the BM and LNs of a single limb was enumerated. In these groups none of the chimeric hosts rejected their limbs. However, hosts of non-irradiated limbs succumbed to GVHD 22.4 ± 0.8 days post-transplantation. In contrast, chimeras that received irradiated ACI hind limbs or hind limbs that had all LNs removed showed no clinical or histological signs of GVHD at 5 months.

The percentage of $\alpha\beta$ TCR⁺ T cells within LNs was approximately 12 times higher than in BM of a single limb. This data indicates that lymphocyte load of LNs, in the CTA hind limb is responsible for lethal GVHD in chimeric host. We conclude that mixed chimeras are susceptible to GVHD when receiving LN bearing grafts. LN removal could be a preventive measure against GVHD.

The role of LNs in adaptive immune responses has been the subject of extensive research. In previous studies, the surgical removal of lymph nodes from rat hind limbs prevented the development of lethal GVHD after allogeneic hind limb transplantation to chimeric recipient rats. The purpose of the study in **chapter 10** was to establish the role of the cellular fraction versus the microenvironment of LNs in the development of GVHD in this model. A rat model for vascularized LN transplantation was first developed (**chapter 9**) and graft-versus-host responses were compared after: 1) naive ACI LN cells were infused into WF rats as chimeric recipients (e.g. [ACI→WF]); 2) vascularized WF lymph nodes were transplanted to syngeneic WF recipients; 3) nonvascularized ACI lymph nodes were transplanted to [ACI→WF] chimeric recipients; 4) vascularized ACI lymph nodes were transplanted to [ACI→WF] chimeric recipients. Transplantation of vascularized ACI lymph nodes to [ACI→WF] chimeric recipient rats resulted in severe and sometimes lethal GVHD. In contrast, neither the infusion of purified ACI LN cells nor the transplantation of nonvascularized LNs led to GVHD in chimeric recipients. We concluded that when introducing allogeneic cells into chimeric recipients, concomitant transplantation of the vascularized LN microenvironment makes a manifest difference between induction and absence of GVHD. This illustrates the important role of the LN microenvironment in adaptive immune responses.

PART III

Composite tissue allotransplantation has recently emerged as a new therapeutic modality to reconstruct major tissue defects to restore form and function to the head and neck region and extremities. In contrast to organ transplantation, which is a widely accepted treatment for end-stage organ failure, some argue that in CTA procedures, the risks posed by the immunosuppressant drugs outweigh the benefits received. Since the risk-versus-benefit ratio for CTA procedures has not yet been defined, the decision as to whether or not to perform these new reconstructive techniques is based on subjective opinions. Objective assessment of desirability and risk accep-

tance would certainly facilitate the decision-making process for these innovative procedures. To this end, we have developed a questionnaire-based instrument (Louisville Instrument for Transplantation [LIFT]) to objectively assess the relative risk that individuals are willing to accept in order to benefit from various CTA procedures. The goal of the work described in **chapter 11** is to validate the LIFT instrument.

In **chapter 12** we used this psychometrically reliable and valid instrument to assess the relative risk that individuals are willing to accept in order to receive the benefits of CTA procedures. We investigated two primary populations of individuals: those who live with the risks of immunosuppression, and healthy individuals. The level of risk acceptance for the seven transplant procedures tested (foot, single hand, double hand, larynx, kidney, hemiface, and full face) showed significant differences in research participants' risk acceptance for the different transplant procedures, but no significant differences between groups. Based on these findings, we concluded that certain CTA procedures convey benefits to recipients that are perceived by subjects, including individuals who live with the risks of immunosuppression, to warrant the risks of these procedures.

Using the LIFT questionnaire we quantitatively assessed the risks versus benefits in facial transplantation (**chapter 13**). Since the surgical techniques necessary to transplant a human face are well established, it is the ethical barriers that pose the greatest challenge to performing routine facial transplantation. Respondents in three study populations (healthy individuals, $n = 150$; organ transplant recipients, $n = 42$; and individuals with facial disfigurement, $n = 34$) were questioned about the extent to which they would trade off specific numbers of life-years, or sustain other costs, in exchange for receiving seven different transplant procedures. It was found that the three populations would accept differing degrees of risk for the seven transplant procedures. Organ transplant recipients were the most risk-tolerant group, while facially disfigured individuals were the least risk tolerant. All groups questioned would accept the highest degree of risk to receive a face transplant compared with the six other procedures. This study presents an empirical basis for assessing risk versus benefit in facial transplantation. In doing so, it provides a more solid foundation upon which to introduce this exciting new reconstructive modality into the clinical arena.

The purpose **chapter 14** is to inform the reader of the major technical, immunological and ethical issues surrounding facial transplantation and to elicit professional discussion from the surgical community.

Technical issues: Facial transplantation would consist of removing facial tissues from a brain dead donor and transplanting it to a recipient to recon-

struct a facial defect. The severely scarred and fibrotic tissue on the recipient's face would be removed and replaced with anatomically and functionally normal tissues, which over a period of 1-2 years would be expected to regain significant facial nerve function and animation. The donor facial tissue will be matched to and patterned from the defect defined by the recipient's deformity. This segment of tissue will include skin, subcutaneous tissue, muscle, and the arteries, veins and nerves necessary to satisfactorily perfuse and innervate the facial musculature of the transplanted facial tissue.

Immunological issues: The most common complications associated with the use of immunosuppressants include increased incidence of infections, mostly CMV, malignancies, such as post transplant lymphoproliferative disease and non-melanoma skin carcinoma, and end-organ toxicity, such as diabetes mellitus or nephrotoxicity. It could be argued that in terms of immunosuppression-related end-organ toxicity, facial transplant recipients will be at an advantage over solid organ recipients since solid organ recipients have often already experienced multiple organ problems from their underlying chronic disease. The immunosuppressive drugs they must take would further damage their already debilitated organs. In the case of facial transplant recipients, serious underlying chronic disease would exclude the patient from transplantation.

Ethical issues: The ethical issues associated with the risks and benefits of performing an innovative procedure of this type will always be present. To assure that facial transplantation moves into the clinical research phase in a thoughtful and well planned manner it is important that teams proposing to perform this procedure establish and follow well-defined ethical guidelines. The role of clinical scientists is to gather as much knowledge as possible about a new treatment from research, clinical experience, professional and public discussion and with this inform the patient and his/her family as best as is possible about the associated risks and benefits. We believe that for a select population of severely disfigured individuals facial transplantation, despite its recognized risks, could provide a better treatment option than current methods. As with all innovative medical advances ultimately it is the patient who must decide whether to be treated.

Transplantation continues to push the frontiers of medicine into domains that summon forth troublesome ethical questions. The recent human facial transplantation is again advancing this frontier. In **chapter 15** we develop criteria that, we maintain, must be satisfied in order to ethically undertake this facial transplant procedure. We draw on the criteria advanced by Dr. Francis Moore in the late 1980s for introducing innovative procedures

in transplant surgery. His criteria included the scientific background of the innovation, the skill and experience of the team ("field strength"), the ethical climate of the institution, and open display and public and professional discussion and evaluation. Furthermore we also insist that human face transplantation must meet all the ethical requirements usually applied to health care research, in particular risk/benefit assessments, informed consent and privacy and confidentiality. We summarize the achievements of transplant surgery to date, focusing in particular on the safety and efficacy of immunosuppressive medications. We also emphasize the importance of risk/benefit assessments that take into account the physical, aesthetic, psychological, and social dimensions of facial disfiguration, reconstruction, and transplantation. Finally, at the time of publication of this manuscript (2004) we maintained that the time had come to move facial transplantation research into the clinical phase. The developments during the past two years showed that in a very short time span research was successfully translated into an exciting clinical reality, moving the horizons of plastic surgery towards a new era.





Summary in Dutch (Nederlandse Samenvatting)

“Composite tissue allotransplantation”, ofwel transplantatie van samengestelde weefsels, zoals een hand, larynx en gelaat wordt sinds kort (experimenteel) klinisch toegespast. Wereldwijd zouden deze transplantaties van samengesteld weefsel een uitkomst kunnen bieden voor mensen met grote weefseldefecten als gevolg van ernstige ongevallen, tumorverwijdering, brandwonden en aangeboren afwijkingen. Hoewel deze transplantaties van samengestelde weefsels een veelbelovende nieuwe behandelingsoptie lijken voor mensen met grote weefseldefecten, zijn er momenteel nog grote nadelen aan verbonden. Patiënten moeten namelijk immuunsuppressiva (medicijnen die de afweer onderdrukken) gebruiken om afstoting van het transplantaat afkomstig van een ander individu te voorkomen. Zelfs de meest moderne immuunsuppressiva hebben bijwerkingen zoals infecties, orgaanschade en het ontstaan van kanker op de lange termijn.

Dit proefschrift bestaat uit drie delen. In **deel I** werden verschillende combinaties van immuunsuppressiva onderzocht in een varkens- en rattenmodel. Met name werd het effect van de immuunsuppressiva op de kwaliteit van het bot onderzocht.

Het gebruik van immuunsuppressiva en de bijbehorende bijwerkingen kan voorkomen worden als de ontvanger van het weefsel immunologisch tolerant zou zijn voor het donorweefsel. Donor specifieke tolerantie kan worden bereikt door het creëren van chimerisme (het bestaan van cellen van twee genetisch verschillende organismen in één individu) in de ontvanger. In een diemodel kan chimerisme worden geïnduceerd door middel van beenmergtransplantatie, waarbij de afweersystemen van donor en ontvanger worden samengevoegd. In **deel II** onderzochten we of chimerisme ook tolerantie kan creëren voor transplantatie van samengestelde weefsels, zoals een achterpoot van een rat. In het bijzonder werd gekeken naar de rol van “graft-versus-host disease” (GVHD). In GVHD valt het immuunsysteem van de donor de ontvanger van het transplantaat aan. In **deel III** richtten we ons op de ethische vragen en de risico-acceptatie met betrekking tot deze controversiële transplantaties. Door de zeer recent verrichte drie gelaatstransplantaties is deze discussie nog betekenisvoller geworden.

DEEL I

In **hoofdstuk 1** werd in een varkensmodel het effect van de immuunsuppressiva cyclosporine-mycophenolate mofetil (MMF)-prednison op de kwaliteit en genezing van bot onderzocht, waarbij een deel van het kuitbeen,

spier en huid als vrije lap werd getransplanteerd naar een gecreëerd donordefect. Deze studie beschrijft dat de gebruikte medicatie niet effectief was om afstoting van het bot te voorkomen. Voorts werd duidelijk dat de botkwaliteit na de transplantatie achteruitging en dat deze medicatie veel bijwerkingen veroorzaakte. We concludeerden dat deze medicamenteuze therapie niet ideaal is voor deze vorm van transplantaties.

In **hoofdstuk 2** werd de medicijn combinatie tacrolimus-MMF-prednison onderzocht in hetzelfde varkensmodel. Uit deze studie bleek dat deze medicatie wel effectief was om afstoting van het transplantaat te voorkomen. De lichte achteruitgang van botkwaliteit die werd gevonden, wordt naar onze mening veroorzaakt door de operatie en niet door de immuun-suppressiva. We beschrijven dat het belangrijk is om de botkwaliteit na transplantatie te controleren.

Aangezien van corticosteroïden (zoals prednison) bekend is dat ze een negatief effect op bot hebben, werd in **hoofdstuk 3** gekeken of de combinatie van tacrolimus en MMF, zonder prednison, ook afstoting zou kunnen voorkomen in een rattenmodel, waarbij een achterpoot werd getransplanteerd. De resultaten toonden aan dat deze combinatie zonder grote bijwerkingen afstoting kan voorkomen.

In **hoofdstuk 4** werd in ditzelfde model de kwaliteit en genezing van bot onderzocht in zowel de getransplanteerde achterpoten als de eigen (niet-getransplanteerde) achterpoten. We concluderen dat de medicatie in dit rattenmodel de botkwaliteit verslechtert.

DEEL II

Zelfs met de meest moderne immuun-suppressiva wordt ongeveer 35% van de getransplanteerde organen binnen 5 jaar afgestoten. Daarom is het induceren van immunologische tolerantie voor getransplanteerd weefsel één van de belangrijkste doelen in transplantatie geneeskunde.

In **hoofdstuk 5** wordt een overzicht gegeven over het induceren van chimerisme en tolerantie, en het omzeilen van de huidige beperkingen.

Hoofdstuk 6 beschrijft een rattenmodel waarbij chimerisme werd geïnduceerd door middel van beenmergtransplantatie. Echter bij transplantatie van een niet-gemanipuleerde donor achterpoot naar een tolerante ontvanger, bleek dat het meegetransplanteerde immuunsysteem de ontvanger afstootte (GVHD). Inactivatie van het immuunsysteem door middel van bestraling van de donor was succesvol om GVHD te voorkomen.

Hoofdstuk 7 beschrijft een rattenmodel waarbij het induceren van chimerisme en de transplantatie van de achterpoot simultaan werden uitgevoerd. Er werd een hoog percentage chimerisme verkregen in combinatie met robuuste tolerantie en volledige overleving van de getransplanteerde achterpoot. In **hoofdstuk 8** werd onderzocht of het beenmerg danwel de lymfeklieren aanwezig in de getransplanteerde rattenachterpoot verantwoordelijk waren voor het ontstaan van GVHD in de ontvanger. Hiertoe werden de lymfeklieren voorafgaand aan de transplantatie uit de achterpoot verwijderd en daarmee werd aangetoond dat de lymfocyten aanwezig in de lymfeklieren de dodelijke GVHD veroorzaakten.

Met deze kennis werd in **hoofdstuk 10** de bijdrage van het micromilieu van getransplanteerde lymfeklieren op het ontstaan van GVHD onderzocht. We beschrijven dat infusie van lymfocyten en niet-gevasculariseerde lymfeklier transplantaties niet resulteerde in GVHD in tegenstelling tot gevasculariseerde lymfeklier transplantatie. Dit illustreert de belangrijke rol van het micromilieu van de lymfeklieren bij immunologische reacties.

In **hoofdstuk 9** wordt het model van vrije gevasculariseerde lymfeklier transplantatie in ratten beschreven.

DEEL III

Transplantaties van samengestelde weefsel met gebruik van immuunsuppressiva om afstoting te voorkomen is controversieel. Anders dan bij levensreddende orgaan transplantaties vindt men de risico's van dergelijke immuunsuppressiva zwaarder wegen dan de voordelen, die een patiënt zou verkrijgen bij reconstructie van weefseldefecten met donorweefsel. Toch zou reconstructie met in vorm en functie identiek donorweefsel een ideale oplossing zijn voor de grote behoefte aan weefsel voor diverse reconstructies. Objectieve beoordeling van de wenselijkheid en de risico-acceptatie van deze innovatieve ingrepen zou de afweging om dergelijke ingrepen op grote schaal te introduceren kunnen vergemakkelijken. Zodoende hebben wij een vragenlijst ontwikkeld (Louisville Instrument for Transplantation [LIFT]) om het relatieve risico, dat individuen zouden willen accepteren om te kunnen profiteren van de voordelen van deze transplantaties, te evalueren. In **hoofdstuk 11** wordt deze vragenlijst gevalideerd.

In **hoofdstuk 12** hebben we deze vragenlijst gebruikt om in twee verschillen groepen de risico-acceptatie voor transplantatie van samengestelde weefsels te bepalen. De vragenlijst werd voorgelegd aan patiënten die im-

muunsuppressiva gebruiken en aan gezonde personen. Er werden zeven (hypothetische) transplantatie procedures getest (voet, hand, twee handen, strottehoofd, nier, partieel gelaat, en volledig gelaat). Het niveau van risico-acceptatie verschilde evident tussen de zeven transplantatie procedures. Tussen de twee groepen proefpersonen bestond echter geen significant verschil. We concluderen dat bepaalde transplantaties van samengesteelde weefsels wel degelijk genoeg voordelen bieden om de risico's van transplantatie en immuunsuppressiva te rechtvaardigen. Met dezelfde vragenlijst werd in **hoofdstuk 13** de afweging tussen de risico's en de voordelen van een gelaatstransplantatie onderzocht. De volgende drie groepen proefpersonen werden onderzocht: 1) gezonde personen, 2) orgaantransplantatie patiënten, en 3) personen met verminkingen aan het gelaat. Orgaantransplantatie patiënten waren bereid het meeste risico te accepteren, terwijl mensen met een verminkt gelaat het minste risico wilden aanvaarden. In de drie groepen was men bereid het meeste risico te aanvaarden voor een gelaatstransplantatie. Dit empirische onderzoek draagt bij aan een meer gefundeerde invoering van gelaatstransplantaties. Het doel van **hoofdstuk 14** is om de technische, immunologische, en ethische aspecten van gelaatstransplantatie te bespreken. Technisch is het goed mogelijk om een gelaat te transplanteren. Het zijn echter de ethische dilemma's die de grootste uitdaging vormen om routinematig gelaatstransplantaties uit te voeren. In **hoofdstuk 15** benadrukken wij dat gelaatstransplantatie uitgevoerd dient te worden volgens de ethische principes van medisch wetenschappelijk onderzoek. We geven een samenvatting van de bereikte resultaten van transplantatie chirurgie, met de nadruk op de veiligheid en doeltreffendheid van immuunsuppressiva. Het belang van een goede afweging tussen voor- en nadelen van gelaatstransplantatie met betrekking tot functionele, cosmetische, psychologische, en sociale aspecten wordt besproken. We concludeerden aan het eind van deze publicatie (2004) dat het gerechtvaardigd was om gelaatstransplantaties klinisch uit te gaan voeren. De ontwikkelingen van de afgelopen twee jaar hebben laten zien dat in korte tijd experimenteel onderzoek heeft geleid tot succesvolle klinische gelaatstransplantaties. Deze ontwikkeling luidt een nieuw tijdperk voor de plastische en reconstructieve chirurgie in.



Questionnaire, Louisville Instrument for Transplantation

LOUISVILLE INSTRUMENT FOR TRANSPLANTATION QUESTIONNAIRE

1. Today's Date: _____
2. Your age: _____
3. Your gender (check box): male female

INSTRUCTIONS

You will be asked to give your opinion on various aspects of seven different (imaginary) clinical scenarios concerning organ and tissue transplantation. The questionnaire is arranged so that it asks you the **exact same questions** for the following (imaginary) clinical transplant scenarios:

- Foot transplant
- Kidney transplant
- Hand transplant
- Double hand transplant
- Larynx (voice box) transplant
- Partial face transplant
- Full face transplant

Most of the questions can be answered by simply circling a number on a sliding scale.

To receive any transplant one must take risks, mainly caused by the antirejection medication she/he must take to avoid rejecting the transplant. Therefore, one must weigh the benefits of receiving a transplant versus the risks of the medication. The following questions are designed to assess your opinion on how much risk you would accept to receive the benefit of each of these different transplants.

To assess your opinion we will ask you to equate the amount of risk you would be willing to accept with the number of years of your life you would be willing to give up to receive a given transplant. We will also ask you to consider how well the transplanted part would have to function and look for you to be happy with it.

The following 5 examples are provided to help you understand how to answer the questions on the next pages. Most of the questions can be answered by simply circling a number from 0 to 10 or from 0% to 100% on a sliding scale (See below).

Study ID: _____ (to be filled by investigator)

EXAMPLE: EAR TRANSPLANTATION

You sustained an injury that left you without your left ear.

Suppose you have 10 years to live. You can have an ear transplanted from a donor that provides some touch sensitivity and better appearance than a prosthesis. However, the antirejection medication you will have to take to prevent the ear from rejecting will cause you to die before the above 10 years you had to live.

QUESTION 1: Choose the maximum number of years (0 to 10 years) you would be willing to **give up** in order to get the ear transplant.

Years you would give up to get transplant (circle one)

Give up NO years	0	<input checked="" type="radio"/> 1	2	3	4	5	6	7	8	9	10	Give up ALL years
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ANSWER 1: Circling number 1, for example, would mean that you are willing to give up only 1 out of 10 years to receive an ear transplant. If you would be willing to give up more years to get an ear transplant, you would circle a higher number.

QUESTION 2: Looking at the same scenario in another way, choose the minimum number of years you would **need to live** (0 to 10 years) to get the ear transplant.

Years you would need to live to get transplant (circle one)

Need to live ALL years	10	<input checked="" type="radio"/> 9	8	7	6	5	4	3	2	1	0	Need to live NO years
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ANSWER 2: If you circle 9, this would mean that you would need to live at least 9 years to get the ear transplant and that you require a high level of safety. If you would need to live less years to get the transplant you would require less safety to get the ear transplant and you would circle a lower number.

QUESTION 3: Looking at the same scenario from another angle, **suppose you will live to be 75 years old.**

What is the **maximum percent of the remaining years** of your life you would give up in order to get an ear transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of your years you would give up).

Maximum percent of your remaining years of life you would give up to get a transplant (circle one)

Give up NO % of years	0%	<input checked="" type="radio"/> 10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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ANSWER 3: If you would choose 10%, this means you would be willing to give up few years to receive an ear transplant. For example, if you are now 30, and will live to 75, giving up 10% of your remaining years (45 years) means you would give up 4.5 years to get an ear transplant.

QUESTION 4: In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted ear. What is the **maximum chance of rejection** you would accept and still get the ear transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	<input checked="" type="radio"/> 10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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ANSWER 4: If you circled 10% this means you would only accept the ear transplant if the chances of losing the ear (by rejection) were very low. (10% means 1 out of 10 chances of rejecting and 90% means 9 out of 10 chances of rejecting).

QUESTION 5: In considering the ear transplant, how important is the effect it will have on your **appearance** (image of being complete) as opposed to function?

Importance of appearance (circle one)

IS NOT important	0%	<input checked="" type="radio"/> 10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	IS important
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ANSWER 5: If you circled 10%, this would mean that appearance is not important to you. If you circled 100% this would mean that appearance is very important to you.

Please read the following scenarios, and consider your responses as if the event in the question really involved you. Try to imagine how you would truly respond in the given scenario.

If you have difficulties answering the following questions, feel free to read the examples again. All questions follow the same format as the examples; all that changes is the transplanted part.

SITUATION: FOOT TRANSPLANT

You sustained an injury that left you without your left foot. You currently use a walking prosthesis that allows full walking movement with a cane, although you lack touch sensitivity and cannot use it for sports such as swimming. (Please see figure 1 in photo booklet for picture of foot amputee.)

1. If you **lost your foot**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. You can have a foot transplanted from a donor that provides less strength, but some touch sensitivity and better appearance than a prosthesis. However, the antirejection medication you will have to take to prevent the foot from rejecting will cause you to die before the 10 years you had to live.

2. Choose the maximum number of years (0 to 10 years) you would be willing to **give up** to get a foot transplant.

Years you would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get the foot transplant.

Years needed to live to accept transplant (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not undergo the transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of the remaining years** of your life you would give up in order to get a foot transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of your years you would give up).

Maximum percent of remaining years of life you would give up to get a transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted foot. What is the **maximum chance of rejection** you would accept and still get the foot transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Maximum chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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SITUATION: FOOT TRANSPLANT

7. Suppose the antirejection medications would reduce your life span by **ONE THIRD**. Choose the **minimum level of improvement** the foot transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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8. In considering this transplant, how important is **function** of the transplanted foot (movement, strength) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important is the **appearance** of the foot (image of being complete) as opposed to function?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the foot transplant**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below **“patient informed consent”** paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a foot transplant?

No Yes

12. If the chance of rejecting the leg were **50% in the first year**, would you still get the foot transplant?

No Yes

13. Who should make the decision about this transplantation? **(Circle one)**

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words explain which considerations would be the most important in your deciding to get, or not to get, a foot transplant?

SITUATION: KIDNEY TRANSPLANT

You sustained an injury that destroyed both of your kidneys. You currently receive dialysis three times per week, for three hours per session. You tolerate dialysis reasonably well, although it is uncomfortable, time-consuming, and limits travel. (Please see figure 2 in photo booklet for picture of renal dialysis patient).

1. If you **lost your kidneys**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. You can have a kidney transplant that will provide most kidney function, without as frequent a need for dialysis. However, the antirejection medication you will have to take to prevent the kidney from rejecting will cause you to die before the above 10 years you have to live.

2. Choose the maximum number of years (0 to 10 years) you would be willing to **give up** to get a kidney transplant.

Years would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get a kidney transplant.

Minimum number of years you would need to live (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not get a kidney transplant under any circumstance []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old.** What is the **maximum percent of remaining years** you would give up to get a kidney transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years of your life you would give up (circle one)

NO % of years you would give up	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	ALL % of years you would give up
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted kidney. What is the **maximum chance of rejection** you would accept and still get the kidney transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. Suppose the antirejection medications would reduce your life span by ONE THIRD. Choose the **minimum level of improvement** the kidney transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: KIDNEY TRANSPLANT

8. In considering this transplant, how important is kidney **function** (freedom from dialysis) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important is **appearance** of having a kidney (instead of having to receive dialysis) as opposed to function?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the kidney transplant**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below “**patient informed consent**” paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a kidney transplant?

No Yes

12. If the chance of rejecting the kidney were **50% in the first year**, would you still get the kidney transplant?

No Yes

13. Who should make the decision about this transplantation? (**Circle one**)

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words explain which considerations would be the most important in your deciding to get, or not to get, a kidney transplant?

SITUATION: HAND TRANSPLANT

You sustained an injury that left you without your left hand (non-dominant hand). You currently use a functioning hook that allows grasping larger objects, although you lack touch sensitivity and cannot use it for finer movements, such as turning pages or handling tissue paper. (Please see figure 3 in photo booklet for picture of hand amputee).

1. If you **lost your hand**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. You can have a hand transplanted from a donor that provides less strength, but some touch sensitivity and better appearance than a prosthesis. However, the antirejection medication you will have to take to prevent the hand from rejecting will cause you to die before the above 10 years you have to live.

2. Choose the maximum number of years (0 to 10 years) you would be willing to **give up** in order to get the hand transplant.

Years would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get the hand transplant.

Minimum number of years you would need to live to accept transplant (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not undergo the hand transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of remaining years** you would give up to get a hand transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years you would give up to get a transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted hand. What is the **maximum chance of rejection** you would accept and still get the hand transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. Suppose the antirejection medications would reduce your life span by **ONE THIRD**. Choose the **minimum level of improvement** the hand transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: HAND TRANSPLANT

8. In considering this transplant, how important is hand **function** (movement, strength) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important is the **appearance** of having a normal hand (image of being complete) as opposed to a functioning hand?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the hand transplant**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below "**patient informed consent**" paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a hand transplant?

No Yes

12. If the chance of rejecting the hand were **50% in the first year**, would you still get the hand transplant?

No Yes

13. Who should make the decision about this transplantation? (**Circle one**)

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words, explain which considerations would be the most important in your deciding to get, or not to get, a hand transplant?

SITUATION: DOUBLE HAND TRANSPLANT

You sustained an injury that left you without hands. You currently use a functioning hook that allows grasping larger objects, although you lack touch sensitivity and cannot use it for finer movements, such as turning pages or handling tissue paper.

1. If you **lost both your hands**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. You can have both hands transplanted from a donor that provides less strength, but some touch sensitivity and better appearance than the prosthesis. However, the antirejection medication you will have to take to prevent the hands from rejecting will cause you to die before the 10 years you had to live.

2. Choose the maximum number of years of life (0 to 10 years) that you would be willing to **give up** in order to get both hands transplanted.

Years would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get both hands transplanted.

Years needed to live to accept transplant (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not undergo the transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of remaining years** you would give up to get a double hand transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years you would give up to get transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted hands. What is the **maximum chance of rejection** you would accept and still get the double hand transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. **Suppose the antirejection medications would reduce your life span by ONE THIRD.** Choose the **minimum level of improvement** the double hand transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: DOUBLE HAND TRANSPLANT

8. In considering this transplant, how important is hand **function** (movement, strength) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important is the **appearance** of the both hands (image of being complete) as opposed to function?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the double hand transplant**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below **“patient informed consent”** paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a double hand transplant?

No Yes

12. If the chance of rejecting both hands were **50% in the first year**, would you still get the hand transplants?

No Yes

13. Who should make the decision about this transplantation? **(Circle one)**

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words, explain which considerations would be the most important in your deciding to get, or not to get, the hand transplants?

SITUATION: LARYNX TRANSPLANT

You sustained an injury that destroyed your larynx (voice box), the part of your throat that controls much of the ability to speak. You have some speech through an electronic device, but it is difficult to use, you must speak very slowly, and even then your voice sounds flat and electronic, like a robot. People have difficulty understanding you.

1. If you **lost your larynx**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. New medical developments allow the possibility of a larynx transplant that provides the opportunity to speak normally. However, the antirejection medication you will have to take to prevent the larynx from rejecting will cause you to die before the 10 years you had to live.

2. Choose the maximum number of years of life (0 to 10 years) that you would be willing to **give up** in order to get the larynx transplant.

Years would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get the larynx transplant.

Years needed to live to accept transplant (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not undergo the transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of remaining years** you would give up to get a larynx transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years you would give up to get transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted larynx. What is the **maximum chance of rejection** you would accept and still get the larynx transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. Suppose the antirejection medications would reduce your life span by **ONE THIRD**. Choose the **minimum level of improvement** the larynx transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: LARYNX TRANSPLANT

8. In considering this transplant, how important is larynx **function** (return of voice) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important is the **appearance** of having a normal voice (image of being complete) as opposed to function?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the larynx transplant**, what do you think **your quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below **“patient informed consent”** paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a larynx transplant?

No Yes

12. If the chance of rejecting the larynx were **50% in the first year**, would you still get the larynx transplant?

No Yes

13. Who should make the decision about this transplantation? **(Circle one)**

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words, explain which considerations would be the most important in your deciding to get, or not to get, a larynx transplant?

SITUATION: PARTIAL FACE TRANSPLANT

You sustained an injury that destroyed the left half of your face. Tissues from other areas of your body have been used to cover the defect. After this treatment the skin looks tight and blotchy, and you lack your left eyebrows, lips and most of your nose. People have difficulty looking at you. (Please see figure 4 in photo booklet for picture of individual with face injury).

1. If you **had such facial disfigurement**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

	Quality of life (circle one)											
WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality

Suppose you have 10 years to live. New medical developments allow the possibility of a partial face transplant. You can have a partial face transplant from a donor that provides a better appearance, including eyebrow, nose and lips. You may not have full touch sensitivity or movement. However, the antirejection medication you will have to take to prevent the partial face transplant from rejecting will cause you to die before the 10 years you had to live.

2. Choose the maximum number of years of life (0 to 10 years) that you would be willing to **give up** in order to get the partial face transplant.

	Years would give up to get transplant (circle one)											
Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years

3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get the partial face transplant.

	Years needed to live to accept transplant (circle one)											
Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years

4. Check here if you would not undergo the transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of remaining years** you would give up to get a partial face transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years you would give up to get transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted partial face. What is the **maximum chance of rejection** you would accept and still get the partial face transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. Suppose the antirejection medications would reduce your life span by ONE THIRD. Choose the **minimum level of improvement** the partial face transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: PARTIAL FACE TRANSPLANT

8. In considering this transplant, how important is facial **function** (movement, sensation) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important would the improvement of your **appearance** be (image of being complete) as opposed to function?

Importance of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. If you **got the partial face transplant**, what would you perceive your **quality of life** to be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below **“patient informed consent”** paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

11. After reading the above would you still want to get a partial face transplant?

No Yes

12. If the chance of rejecting the partial face were **50% in the first year**, would you still get the partial face transplant?

No Yes

13. Who should make the decision about this transplantation? **(Circle one)**

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

14. In a few words, explain which considerations would be the most important in your deciding to get, or not to get, a partial face transplant?

SITUATION: FULL FACE TRANSPLANT

You sustained an injury that destroyed your whole face. Tissues from other areas of your body have been used to cover the defect. After this treatment the skin looks tight and blotchy, and you lack your eyebrows, lips and your nose. People have difficulty looking at you.

1. If you **had such facial disfigurement**, what do you think your **quality of life** would be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Suppose you have 10 years to live. New medical developments allow the possibility of a full face transplant. You can have a full face transplant from a donor that provides a better appearance, including eyebrow, nose and lips. You may not have full touch sensitivity or movement. However, the antirejection medication you will have to take to prevent the full face transplant from rejecting will cause you to die before the 10 years you had to live.

2. Choose the maximum number of years of life (0 to 10 years) that you would be willing to **give up** in order to get the full face transplant.

Years would give up to get transplant (circle one)

Give up NO years	0	1	2	3	4	5	6	7	8	9	10	Give up ALL years
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3. Looking at the same scenario from another angle, choose the minimum number of years you would **need to live** (0 to 10 years) to get the full face transplant.

Years needed to live to accept transplant (circle one)

Need to live ALL years	10	9	8	7	6	5	4	3	2	1	0	Need to live NO years
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4. Check here if you would not undergo the transplant under any circumstances []

5. Looking at the same scenario from another angle, **suppose you will live to be 75 years old**. What is the **maximum percent of remaining years** you would give up to get a full face transplant? (It is not necessary to calculate the exact number of years, just tell us your feeling of what percentage of years you would give up).

Maximum percent of remaining years you would give up to get transplant (circle one)

Give up NO % of years	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Give up ALL % of years
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6. In all transplants there is always a chance of rejection, which could cause the complete loss of the transplanted face. What is the **maximum chance of rejection** you would accept and still get the full face transplant? (90% means you would accept a high chance of rejection and 10% means you would accept a low chance of rejection)

Chance of rejection (circle one)

LOW chance of rejection	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	HIGH chance of rejection
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7. Suppose the antirejection medications would reduce your life span by **ONE THIRD**. Choose the **minimum level of improvement** the full face transplant would have to provide for you to accept it? (100% means maximal improvement (identical to normal) and 0% means no improvement).

Minimum level of improvement (circle one)

Minimal improvement	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	Maximal improvement
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SITUATION: FULL FACE TRANSPLANT

8. In considering this transplant, how important is facial **function** (movement, sensation) as opposed to appearance?

Importance of function (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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9. In considering this transplant, how important would it be that the transplant gives you full capacity for facial expression?

Importance of facial expression (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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10. In considering this transplant, how important would the improvement of your appearance be (image of being complete) as opposed to function?

Importance of improvement of appearance (circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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11. In considering this transplant, how important would it be that the transplant gives you the same facial appearance as before the injury (as opposed to looking like a different person after transplantation)?

(circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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12. Check here if looking like a different person after transplantation would prevent you from undergoing the transplant []

13. In considering this transplant, how important would it be that the transplant gives you esthetically pleasing (pretty or handsome) features?

(circle one)

Not important	0	1	2	3	4	5	6	7	8	9	10	Extremely important
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14. If you **got the full face transplant**, what would you perceive your **quality of life** to be (include your personal, social and occupational quality)?

Quality of life (circle one)

WORST quality	0	1	2	3	4	5	6	7	8	9	10	BEST quality
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Please read the below **“patient informed consent”** paragraph.

After your transplant you will need to take antirejection medications for the rest of your life. The risks of these medications include: nausea, vomiting, diarrhea, constipation, weight gain, dizziness, urinary tract infections, hypertension, diabetes, kidney failure, headaches, liver toxicity; tumors of lymph glands, skin or major organs; atherosclerosis, osteoporosis, bacterial or viral infections. Even taking your medication, your body may still reject the transplant and it will need to be surgically removed. If any life threatening complications arise from the antirejection medications, these medications will need to be discontinued and the transplant will need to be surgically removed.

15. After reading the above would you still want to get a full face transplant?

[] No [] Yes

16. If the chance of rejecting the full face were **50% in the first year**, would you still get the full face transplant?

[] No [] Yes

SITUATION: FULL FACE TRANSPLANT

17. Who should make the decision about this transplantation? (Circle one)

1. Patient
2. Physician
3. Shared decision between patient and physician
4. Shared decision between patient and relatives
5. Shared decision between patient, relatives and physician

18. In a few words, explain which considerations would be the most important in your deciding to get, or not to get, a full face transplant?

19. Please **rank** these 3 facial features in order (1-2-3) from **most** important (1) to **least** important (3).

Use each number only once (1,2,3).

- __ Movement of forehead and eyebrows
- __ Movement of eyelids and corners of eyes
- __ Movement of lips

20. Please **rank** these 3 facial features in order (1-2-3) from **most** important (1) to **least** important (3).

Use each number only once (1,2,3).

- __ Appearance
- __ Movement
- __ Touch sensitivity

21. Please **rank** these 7 facial features in order (1-2-3-4-5-6-7) from **most** important (1) to **least** important (7).

Use each number only once (1,2,3,4,5,6,7).

- __ Appearance of forehead
- __ Appearance of eyebrows
- __ Appearance of eyelids
- __ Appearance of cheeks
- __ Appearance of nose
- __ Appearance of lips
- __ Appearance of chin

22. Do you think that a full face transplant will make your face look like the face of the donor?

No Yes

23. If so, would you still get the full face transplant?

No Yes

PLEASE CIRCLE THE BEST ANSWER TO THE BELOW QUESTIONS

- 1. Your race / ethnicity:** 1 = Caucasian/ White (non-Hispanic) 4 = Hispanic/Latino Origin
2 = Asian/Pacific Islander 5 = Native American/Alaskan Native
3 = Black or African-American 6 = Other, specify: _____

- 2. Your current marital status:** 1 = Never Married 4 = Separated
2 = Married 5 = Divorced
3 = Living with partner in committed relationship 6 = Widowed

- 3. Your current living arrangement:** 1 = Alone
2 = With other adult(s), no dependents
3 = With other adult(s) and dependents*
4 = With dependents* only
5 = In an institution or retirement home

*Dependents can include children, elderly, or the infirm

- 4. How many children do you have? (Biological or legally adopted):** _____

- 5. Religious affiliation:** 1 = Catholic 4 = Muslim
2 = Protestant 5 = No religious affiliation
3 = Jewish 6 = Other, please specify: _____

- 6. What is the highest grade in school that you completed?**
1 = 1-8 grades 5 = Junior College Degree/AA
2 = 9-11 grades 6 = College Degree (BA/BS)
3 = High School Grad/GED 7 = Some post-college work
4 = Some college 8 = Advanced degree (MA, PhD, MD)

- 7. Health insurance status:** 1 = Medicaid 5 = HMO or other limited provider
2 = Medicare only 6 = Private health insurance (PPO)
3 = Medicare +Supplemental 7 = No insurance
4 = Disability insurance 8 = Other (please specify): _____

- 8. What is your primary occupational status at this time?**
1 = Homemaker 5 = On leave of absence
2 = Unemployed 6 = Full-time employed
3 = Retired 7 = Part-time employed
4 = On Disability 8 = Student

- 9. What is your current/most recent occupation?**
1 Professional, Technical, & Related (teacher/professor, nurse, lawyer, physician, engineer)
2 Manager, Administrator, or Proprietor (sales manager, real estate agent, or postmaster)
3 Clerical & Related (secretary, clerk, mail carrier)
4 Sales (salesperson, demonstrator, agent, broker)
5 Service (police, cook, hairdresser)
6 Skilled Crafts & Related (carpenter, repairer, telephone line worker)
7 Equipment or Vehicle Operator & Related (driver, railroad brakeman, sewer worker)
8 Laborer (helper, longshoreman, warehouse worker)
9 Farmer (owner, manager, operator, tenant)
10 Member of the military
11 Homemaker
12 Other, please describe: _____
13 Student

- 10. What is your family's household income?** 1= less than 20,000 4= between 60,000 and 80,000
2= between 20,000 and 40,000 5= more than 80,000
3= between 40,000 and 60,000

- 11. Do you have any significant chronic health problems, such as heart disease, diabetes, arthritis, etc.?**
[] No [] Yes If yes, please explain: _____

If you have received a transplant, answer questions 12-15, which refer to the time of your transplant. If you have not received a transplant skip questions 12-15.

12. When did you receive a transplant? What type of transplant did you receive?

Since your transplant: Do you think your transplant significantly contributed to any of the following?

- | | | |
|------------------------------------|-----------------------------|------------------------------|
| 13. Marital separation or divorce | <input type="checkbox"/> No | <input type="checkbox"/> Yes |
| 14. Loss of employment opportunity | <input type="checkbox"/> No | <input type="checkbox"/> Yes |
| 15. Improved quality of life | <input type="checkbox"/> No | <input type="checkbox"/> Yes |

⇒ **Please continue here.** Read the following instructions and use the provided scale to mark the appropriate number. Please note that the scale changes per group of questions.

INSTRUCTIONS: Use this list of common human **feelings** to describe YOURSELF as you generally are, compared to others of your sex and age. Use this scale to say how accurate each statement is about YOU. Mark the appropriate number on your answer sheet.

1	2	3	4	5	6	7
Not at all like me	Sometimes not like me	In equal amounts	Sometimes like me			Very much like me

- ___ 1. I feel I'm a person of worth, at least on an equal basis with others.
- ___ 2. I feel that I have a number of good qualities.
- ___ 3. All in all, I am inclined to feel that I am a failure.
- ___ 4. I am able to do things as well as most other people.
- ___ 5. I feel I do not have much to be proud of.
- ___ 6. I take a positive attitude towards myself.
- ___ 7. On the whole, I am satisfied with myself.
- ___ 8. I wish I could have more respect for myself.
- ___ 9. I certainly feel useless at times.
- ___ 10. At times I think I am no good at all.

INSTRUCTIONS: Use this list of common human **characteristics** to describe YOURSELF. Describe yourself as you are generally or typically, as compared to other persons of your sex and approximate age. Use the scale to say how accurate each trait is about YOU. Mark the appropriate number on your answer sheet.

- ___ 11. Generally, I'm not very aware of myself
- ___ 12. I don't find it hard to talk to strangers.
- ___ 13. I feel anxious when I speak in front of a group.
- ___ 14. I get embarrassed very easily.
- ___ 15. I have trouble working when someone is watching me.
- ___ 16. I never scrutinize myself.
- ___ 17. Reflect about myself a lot.
- ___ 18. I sometimes have the feeling that I'm off somewhere watching myself.
- ___ 19. I usually worry about making a good impression.
- ___ 20. I'm alert to changes in my mood.
- ___ 21. I'm always trying to figure myself out.
- ___ 22. I'm aware of the way my mind works when I work through a problem.
- ___ 23. I'm concerned about my style of doing things.
- ___ 24. I'm concerned about the way I present myself.
- ___ 25. I'm concerned about what other people think of me.
- ___ 26. I'm constantly examining my motives.
- ___ 27. I'm generally attentive to my inner feelings.
- ___ 28. I'm often the subject of my own fantasies.
- ___ 29. I'm self-conscious about the way I look.
- ___ 30. I'm usually aware of my appearance.
- ___ 31. It takes me time to overcome my shyness in new situations.
- ___ 32. Large groups make me nervous.
- ___ 33. One of the last things I do before I leave my house is look in the mirror.

INSTRUCTIONS: Use this list of common human **feelings** to describe YOURSELF. Describe yourself as you are generally or typically, as compared to other persons of your sex and approximate age. Use this scale to say how accurate each statement is about YOU. Mark the appropriate number on your answer sheet.

1	2	3	4	5	6	7	
Strongly agree	Sometimes agree			In equal amounts	Sometimes disagree		Strongly disagree

- ___34. What I look like is an important part of who I am.
- ___35. What's wrong with my appearance is one of the first things that people will notice about me.
- ___36. One's outward physical appearance is a sign of the character of the inner person.
- ___37. If I could look just as I wish, my life would be much happier.
- ___38. If people knew how I really look, they would like me less.
- ___39. By controlling my appearance, I can control many of the social and emotional events in my life.
- ___40. My appearance is responsible for much of what has happened to me in my life.
- ___41. I should do whatever I can to always look my best.
- ___42. Aging will make me less attractive.
- ___43. For women: To be feminine, a woman must be as pretty as possible.
- ___44. For men: To be masculine, a man must be as handsome as possible.
- ___45. The media's messages in our society make it impossible for me to be satisfied with my appearance.
- ___46. The only way I could ever like my looks would be to change what I look like.
- ___47. Attractive people have it all.
- ___48. Homely people have a hard time finding happiness.
- ___49. People often tell me I look 100 years old.

Using the scale below as a guide, choose a number to indicate how much you agree with each statement.

1	2	3	4	5
Very true	Somewhat true			Not true

- ___50. My first impressions about people usually turn out to be right.
- ___51. It would be hard for me to break any of my bad habits.
- ___52. I don't care to know what other people really think of me.
- ___53. I have not always been honest with myself.
- ___54. I always know why I like things.
- ___55. I don't know what my major strengths and weaknesses are.
- ___56. Once I've made up my mind, other people can seldom change my opinion.
- ___57. I am not a safe driver when I exceed the speed limit.
- ___58. I am fully in control of my own fate.
- ___59. It's hard for me to shut off a disturbing thought.
- ___60. I never regret my decisions.
- ___61. I sometimes lose out on things because I can't make up my mind soon enough.
- ___62. The reason I vote is because my vote can make a difference.
- ___63. My parents were not always fair when they punished me.
- ___64. I am a completely rational person.
- ___65. I rarely appreciate criticism.
- ___66. My solutions to problems are original and effective.
- ___67. I have sometimes doubted my ability as a sex partner.
- ___68. It's all right with me if some people happen to dislike me.
- ___69. I don't always know the reasons why I do the things I do.
- ___70. I sometimes tell lies if I have to.

Using the scale below as a guide, choose a number to indicate how much you agree with each statement.

0	1	2	3	4	5
Strongly disagree		Disagree	Neutral	Agree	Strongly agree

- ___ 71. In uncertain times, I usually expect the best.
- ___ 72. It's easy for me to relax.
- ___ 73. If something can go wrong for me, it will.
- ___ 74. I'm always optimistic about my future.
- ___ 75. I enjoy my friends a lot.
- ___ 76. It's important for me to keep busy.
- ___ 77. I hardly ever expect things to go my way.
- ___ 78. I don't get upset too easily.
- ___ 79. I rarely count on good things happening to me.
- ___ 80. Overall, I expect more good things to happen to me than bad

81. How physically attractive are you?

- 1 = extremely unattractive
- 2 = very unattractive
- 3 = somewhat unattractive
- 4 = average
- 5 = somewhat attractive
- 6 = very attractive
- 7 = extremely attractive

Below are some statements with which you may agree or disagree. Use the scale below to show your agreement with each item. Place the number on the line for that item. Please be open and honest in your answers.

1	2	3	4	5	6	7
Strongly disagree	Disagree	Slightly disagree	Neither agree or disagree	Slightly agree	Agree	Strongly agree

- ___ 82. In most ways my life is close to my ideal.
- ___ 83. The conditions of my life are excellent.
- ___ 84. I am satisfied with my life.
- ___ 85. So far I have gotten the important things I want from life.
- ___ 86. I am generally pleased with the life I lead.
- ___ 87. The conditions of my social life are excellent.
- ___ 88. I am satisfied with my social life.
- ___ 89. So far I have gotten the important things I want from my social life.
- ___ 90. I am generally pleased with the social life I lead.
- ___ 91. In most ways my sex life is close to my ideal.
- ___ 92. The conditions of my sex life are excellent.
- ___ 93. I am satisfied with my sex life.
- ___ 94. So far I have gotten the important things I want from my sex life.
- ___ 95. I am generally pleased with the quality of my sex life.
- ___ 96. In most ways my actual self is close to my ideal life.
- ___ 97. As an individual I consider myself excellent.
- ___ 98. I am satisfied with my person or self as an individual.
- ___ 99. So far I have gotten the important things I want from myself.
- ___ 100. I am generally pleased with myself as an individual.
- ___ 101. In most ways my actual physical appearance is close to my ideal physical appearance.
- ___ 102. I consider my physical appearance excellent.
- ___ 103. I am satisfied with my physical appearance.
- ___ 104. There is nothing about my physical appearance that I would like to change.
- ___ 105. I am generally pleased with my physical appearance.

The questions below pertain to your current "immediate" family not your "extended" family.

- ___ 106. In most ways my family life is close to my ideal.
- ___ 107. The conditions of my family life are excellent.
- ___ 108. I am satisfied with my family life.
- ___ 109. So far I have gotten the important things I want from my family life.
- ___ 110. I am generally pleased with the quality of my family life.

1	2	3	4	5	6	7
Strongly disagree	Disagree	Slightly disagree	Neither agree or disagree	Slightly agree	Agree	Strongly agree

DO YOU GO TO SCHOOL? No Yes **If not, skip the next 5 questions**

(Use scale above)

- ___ 111. The education I get at school is great.
- ___ 112. I like or respect the other students at school.
- ___ 113. I am satisfied with my classes.
- ___ 114. So far I have learned the important things I wanted at school.
- ___ 115. I am generally pleased with the quality of my teacher.

DO YOU HAVE A JOB? No Yes **If not, skip the next 10 questions**

(Use scale above)

- ___ 116. The chance for advancement on my job is good.
- ___ 117. I like the company policies and practices.
- ___ 118. Like or respect my coworkers.
- ___ 119. I am pleased with the praise I get for doing a good job.
- ___ 120. I am given enough freedom to use my own judgment.
- ___ 121. I like the way my job provides for steady employment.
- ___ 122. My boss handles his or her employees well.
- ___ 123. I am happy with the competence of my supervisor.
- ___ 124. The working conditions of my job are excellent.
- ___ 125. Overall, I am satisfied with my job.

Are you in an "exclusive" relationship?

<input type="checkbox"/> YES ↓ If you checked this box, please answer the 5 questions below based on your current relationship	<input type="checkbox"/> NO (But I have been in the past) ↓ If you checked this box, please answer the 5 questions below based on your past relationship	<input type="checkbox"/> NO (And I have not been in the past) ↓ If you checked this box, you may stop here
--	--	---

(Use scale above)

- ___ 126. In most ways my relationship/marriage is close to my ideal.
- ___ 127. The conditions of my relationship/marriage are excellent.
- ___ 128. I am satisfied with my relationship/marriage.
- ___ 129. So far I have gotten the important things I want from my relationship/marriage.
- ___ 130. I am generally pleased with the quality of my relationship/marriage.

1. Approximately how long did it take to answer this questionnaire? _____ Minutes

2. How difficult was it to complete this questionnaire?

<input type="checkbox"/> Not at all	<input type="checkbox"/> Slightly	<input type="checkbox"/> Moderately	<input type="checkbox"/> Very	<input type="checkbox"/> Extremely
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END OF QUESTIONNAIRE

Remarks you wish to make:

PHOTO BOOKLET LIFT QUESTIONNAIRE

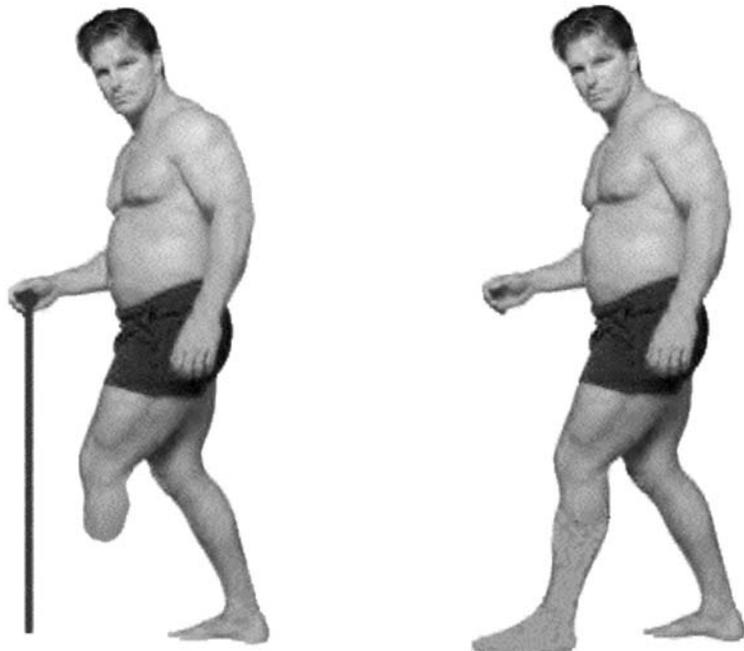


Figure 1.

Left. Individual after left foot amputation

Right. Individual after left foot transplantation



Figure 2.

Individual receiving renal dialysis

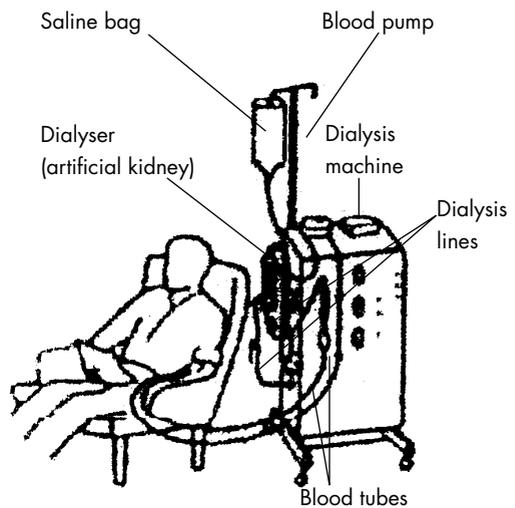




Figure 3.
Left. Individual after left hand amputation
Right. Individual after left hand transplantation

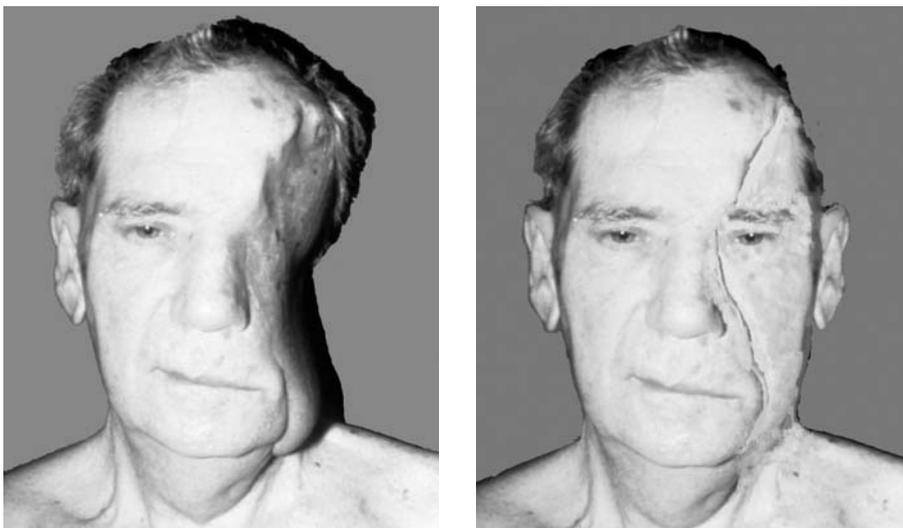


Figure 4.
Left. Individual after left face injury
Right. Individual after left face transplantation



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DANKWOORD MARIEKE EN PASCAL

Het maken van een duo-proefschrift doe je niet met z'n tweeën. Het duurde 7 jaar. In die tijd hebben heel veel mensen op uiteenlopende wijze een bijdrage geleverd.

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Department of Pathology and Laboratory Medicine: R. Fernandez-Botran,
PhD.

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Barnaby, thank you very much for the beautiful cover illustration and for providing us with so many options to choose from!

(VERVOLG) MARIEKE

Beste Pascal, collega promovendus, ondanks een wat hobbelig begin in Louisville ben ik blij dat we dit project samen hebben afgesloten. Het was prettig met je op de eindstreep af te gaan waarbij we volledig op één lijn zaten en veel gelachen hebben. Dank je!

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(VERVOLG) PASCAL

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JC Odiamo, Bas, Merijn, Ward, Wessel, bedankt voor het samen groot worden¹.

(¹ Plagiaat proefschrift J.W. Ganzevoort, Plasma volume expansion in early-onset hypertensive disorders of pregnancy, september 2007)

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Curriculum Vitae

CURRICULUM VITAE MARIEKE VOSSEN

Marieke Vossen werd geboren op 13 maart 1973 te Son en Breugel. Na het behalen van het eindexamen VWO op het Lorentz-Lyceum te Eindhoven, ging zij in 1991 geneeskunde studeren aan de Vrije Universiteit te Amsterdam. Tijdens haar studie verrichtte zij haar eerste onderzoeks-stappen op het gebied van de plastische chirurgie. Voor aanvang van de co-schappen werd nog onderzoek verricht in Yogyakarta, Indonesië. De co-schappen werden afgesloten in Kenia in het St. Elisabeth Hospital te Mukumu met een oudste-coschap heelkunde. Na het behalen van het artsexamen in 1999, werkte zij als AGNIO heelkunde in het OLVG te Amsterdam. In 2000 kreeg zij de mogelijkheid om samen met collega Pascal Brouha onderzoek te doen in de Plastic Surgery Research Laboratories te Louisville, KY, USA (Director, J.H. Barker, MD, PhD). Zij werkte daar onder andere aan het Composite tissue allotransplantation project, hetgeen geleid heeft tot deze duo-promotie. In januari 2003 begon zij met de vooropleiding heelkunde in het Kennemer Gasthuis te Haarlem (opleider dr. H.L.F. Brom). De opleiding tot plastisch chirurg werd in januari 2005 vervolgd bij de vakgroep Plastische, Reconstructieve en Hand Chirurgie van het Universitair Medisch Centrum Utrecht (opleider en hoofd Prof. dr. M. Kon). Op dit moment is zij als AIOS werkzaam bij de maatschap Plastische Chirurgie in het St. Antonius Ziekenhuis te Nieuwegein (opleider dr. A.B. Mink van der Molen). Marieke woont samen met Teun Bruijn, zij hebben een zoon Jibbe en verwachten weer een kindje.

CURRICULUM VITAE PASCAL BROUHA

Pascal Charles Raymond Brouha werd geboren op 5 juli 1974 te Lieshout. Na het behalen van het Gymnasium Bèta diploma aan het Lorentz Lyceum te Eindhoven, studeerde hij vanaf 1992 Geneeskunde aan de Universiteit Utrecht. Tijdens zijn studie was hij betrokken bij onderwijs als student-assistent anatomie en werd een stage gelopen in het Tribhuvan University Teaching Hospital, Kathmandu, Nepal. Voorts werd wetenschappelijk onderzoek verricht bij de vakgroep Plastische, Reconstructieve en Hand Chirurgie in het Universitair Medisch Centrum Utrecht (hoofd prof. dr. M. Kon). Na het behalen van het doctoraal examen verrichtte hij onderzoek in de Wound Healing Research Unit, University of Wales College of Medicine, Cardiff (hoofd prof. K.G. Harding). Aansluitend werden de co-schappen ondermeer doorlopen in Utrecht, Salvador en Tunbridge Wells. Eind 1999 werd het artsdiploma behaald. Direct daarna is hij als research fellow verbonden geweest aan de Plastic Surgery Research Laboratories, Department of Surgery, University of Louisville, Louisville, KY, USA (hoofd J.H. Barker, M.D., Ph.D.). Gedurende twee jaar heeft hij hier, o.a. samen met collega Marieke Vossen, onderzoek verricht naar diverse aspecten van transplantatie van samengestelde weefsels in het Composite Tissue Allotransplantation project, waarvan de resultaten worden beschreven in dit proefschrift. In november 2002 begon hij met de vooropleiding heelkunde in het Medisch Centrum Leeuwarden (opleider dr. W.J.H.J. Meijerink). In januari 2005 werd begonnen met de vervolgopleiding plastische chirurgie bij de vakgroep Plastische, Reconstructieve en Hand Chirurgie in het Universitair Medisch Centrum Utrecht (opleider en hoofd prof. dr. M. Kon). In 2006 is hij één jaar als AIOS werkzaam geweest bij de maatschap Plastische Chirurgie in het St. Antonius Ziekenhuis te Nieuwegein (opleider dr. E. Laban).

Pascal is getrouwd met Giza Passos de Cerqueira e Silva.