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EXPERIMENTAL MODELS IN THE PRIMATE FOR RECONSTRUCTIVE SURGERY UTILIZING VASCULARIZED FREE TISSUE TRANSPLANTS WITH NERVE REPAIR

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DEDICATION

To my husband for everything,

to my parents for what I am and what I am not,

to the animals for their sacrifice,

to my patients who keep my feet on the ground,

to wishes, hopes and dreams that allow us to soar,

and to my teachers who have shown me how...

ABSTRACT

The primary goal of reconstructive surgery is the restoration of function and quality of life to patients with congenital or acquired deformities. Unfortunately, severe limitations for reconstruction occur in cases where there is insufficient or inadequate donor tissue resulting in serious morbidity. Successful transplantation of comparable tissue from donors would provide an ideal solution.

Advances in immunosuppression, particularly the discovery of Cyclosporin A, have made successful transplantion of tissue for reconstructive purposes a realistic goal. The requirement for innervation in certain types of reconstructive tissue transplants differs significantly from clinical transplants undertaken until now. Despite several reports of successful long-term survival of adult rat limb transplants using Cyclosporin A, concern has been expressed as to the significance of this animal model in view of the ease with which induced tolerance is possible in rats. The aims of this project were therefore to: 1) successfully design two models of reconstructive tissue transplants in the primate, one with a purely sensory nerve supply, the other a mixed sensory and motor supply and 2) achieve long enough survival for reinnervation to have occurred, assuming it can take place in the presence of the immunosuppressants.

A neurovascular free flap comprised of the entire soft tissue coverage of the second digit and a hand transplant model were successfully designed in the baboon (Papio hamadryas anubis). Seven transplanted neurovascular free flaps and four hand transplants were undertaken. High dose Cyclosporin A was found to be necessary to prevent rejection. The therapeutic range for serum trough levels of the drug in these models was 800-1500 ng/mL. Steroids proved to be a necessary part of the immunosuppressive

regime. Nine out of 11 transplants survived to or beyond 4 months. Average survival for the transplanted neurovascular free flaps was 196 days, ranging from 122-413 days, and 148.5 days for the hand transplants with the longest remaining viable at 311 days. In most cases, the end point was determined by the date for evaluation of reinnervation by our neurophysiologist colleagues and not loss of the transplant due to rejection.

Although the original goals of the project had been achieved, it was not without considerable effort and difficulty. Only 3 out of 11 transplants survived with little or no signs of rejection. All others had significant episodes of rejection, most of which were successfully reversed or controlled by using our rejection protocol. In addition, all animals, to varying degrees, demonstrated some of the following side effects: anorexia, anemia, gingival hyperplasia, hepatotoxicity, hirsutism, lymphoma, nephrotoxicity, subcutaneous or intramuscular abscesses and tremors.

While two models for reconstructive transplantation in the primate have been successfully designed, further work in the field of immunosuppression will be necessary before they can be reproduced with ease and before their application in the human can even be considered. These models have nevertheless already been used by our neurophysiologist colleagues to demonstrate that reinnervation of such tissues does occur in the presence of Cyclosporin A.

RESUME

La chirurgie de reconstruction a pour but la restauration de la fonction et l'amélioration de la qualité de vie des patients atteints de déformation congénitale ou acquise. Malheureusement les résultats sont très limités lorsque la quantité ou la qualité des tissus disponibles n'est pas adéquate. Les patients, atteints d'une morbidité significative, s'amélioreraient beaucoup si les tissus en question pouvaient être transplantés avec succès.

Les découvertes en immunosuppression, particulièrement celle de la Cyclosporine A, ont rendu possible la notion de transplantation à des fins de reconstruction. Les besoins d'innervation pour certains types de greffons utilisés pour la reconstruction font contraste avec les transplantations cliniques faites jusqu'à date. Bien que plusieurs rapports font état de la survie à long terme de membres transplantés chez le rat adulte à l'aide de la Cyclosporine A, des critiques ont été exprimées vis-à-vis ce modèle animal étant donné la facilité à provoquer la tolérance immune chez le rat. Les buts de ce projet étaient donc de: 1) réussir à mettre au point deux modèles de transplantation à des fins de reconstruction, le premier avec une innervation sensitive, le second avec une innervation combinée motrice et sensitive et 2) atteindre un taux de survie à long terme pour permettre la réinnervation, assumant que cette dernière soit possible malgré la présence des immunosuppresseurs.

Les deux modèles mis au point avec succès chez le babouin (Papio hamadryas anubis) étaient premièrement le lambeau libre neurovascularisé comprenant l'ensemble des tissus mous recouvrant l'index et deuxièmement la transplantation de main au niveau de l'avant bras distal. Sept lambeaux libres neurovascularisés et quatre mains ont été transplantés. De fortes doses de Cyclosporine A ont été nécessaires pour prévenir le rejet. En effet le niveau

sérique pre-dose thérapeutique de ce médicament pour ces transplantations était de 800-1500 ng/mL. Les stéroïdes se sont avérés un élément indispensable de ce protocole d'immunosuppression. Neuf des 11 transplants ont survécu 4 mois et plus. Les lambeaux libres neurovascularisés ont survécu entre 122 et 413 jours, pour une moyenne de 196 jours. La moyenne de survie des mains transplantées a été de 148.5 jours, dont une encore viable à 311 jours. Dans la plupart des cas, la date finale était déterminée non pas par la perte du greffon par rejet mais plutôt par la date de l'évaluation de la reinnervation par nos collègues neurophysiologistes.

Ce n'est pas sans effort et de nombreuses difficultés que nous avons pu atteindre nos objectifs originaux. Seulement 3 des 11 greffons ont survécu sans signe significatif de rejet. Tous les autres ont subi des épisodes de rejet, pour la plupart, renversés ou tout au moins controlés, avec notre protocole. En plus, tous les animaux, a un degré plus ou moins sevère, ont montré des effets secondaires: abcès souscutanés ou intramusculaires, anorexie, anémie, hépatotoxicité, hirsutisme, hyperplasie gingivale, lymphome, néphrotoxicité, et tremblements.

Bien que deux modèles de transplantation à des fins de reconstruction chez le primate ont été développés avec succès, des progrès en immunosuppression seront nécessaires avant que ces modèles puissent être reproduit avec facilité et avant même de penser répéter l'expérience chez l'humain. Ces modèles ont malgré tout été utilisés par nos collègues neurophysiologistes pour démontrer que la réinnervation de tels tissus est possible en présence de la Cyclosporine A.

ACKNOWLEDGEMENTS

Nothing in the world exists in a vacuum,
All is interconnected with the rest of the universe,
Were that connection to break,
Everything would vanish...

E.P.E. 1989

I wish to express my sincere gratitude to Dr. Rollin K. Daniel for believing in me and for giving me the opportunity to prove that I too can transform dreams into reality, and to Dr. Carolyn L. Kerrigan for being an inspiration in my professional life.

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guided me to others with the right solution to every obstacle, and to the laboratories that processed innumerable tests as well as those who accepted responsibility in order to help make things happen. Although naming all these individuals without omitting any would be impossible, two people must not go unmentioned, Dr. Ronald D. Guttmann, Director of the Transplantation Service, Royal Victoria Hospital (Montreal, Quebec, Canada) who guided our choice of immunosuppressants and helped develop our rejection protocol, and Dr. R. Clark Sharp, Assistant Scientist in the Department of Genetics, Southwest Foundation for Biomedical Research (San Antonio, Texas, U.S.A.) who undertook the histocompatibility testing of six of our animals.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEFINITIONS AND ABBREVIATIONS

All definitions are specific to their meaning intended in this thesis.

Allele: various genes existing in the species capable of occupying the same gene locus and which in an individual can only be of maximum two different types (one on each coupled chromosome).

Allo-: prefix indicating the genetic dissimilarity within the same species of the word which it modifies from that of the individual in question (e.g., allograft, allo-antibody, allo-ascitic fluid, allo-transplantation, allo-antigen, etc.).

Allogenic graft: allograft.

Allograft: same as an autograft only transplanted to another genetically dissimilar recipient of the same species.

Antibody: immunoglobulin produced by B-lymphocytes generally in response to antigen stimulation.

Antigen: foreign material recognizable by the immune system which normally responds in order to rid the individual of its presence.

Autograft: an anatomic structure, organ or tissue(s) detached and transferred from and applied or reattached to the same individual whether or not immediate vascular repair is undertaken.

Autosome: chromosome other than a sex chromosome.

Axial pattern flap: a flap directly supplied by a longitudinally running vascular pedicle.

B-cell: B-lymphocyte.

B-lymphocyte: non-thymus-dependent lymphocyte resembling the bursa-derived lymphocytes in birds.

°C: degree(s) Celsius.

Class I antigens: HLA -A, -B, and -CW.

Class II antigens: HLA-D (subdivided into D_R or DR, D_Q, and D_P).

cm: centimeter(s).

CyA: Cyclosporin A.

Degloving a digit: removing the entire skin / soft tissue coverage with its neurovascular supply and leaving behind the osteo-tendinous structures.

Donor: individual from whom the anatomic structure, organ or tissue(s) is taken for transplantation.

Epineurium: outermost connective tissue sheath of a nerve.

Eponychium: skin and nail producing tissue immediately proximal to the visible nail and covering the nail root.

First set rejection: rejection of an organ or tissue(s) in a recipient never having been exposed to the specific set of foreign antigens (unprimed recipient).

Flap: tissue(s) transplanted or transferred with immediate repair of, or without sectioning its vascular supply.

Gene locus: specific position on a chromosome.
Glabrous skin: non-hair bearing skin (palmar and plantar surfaces).
Graft: tissue detached and transplanted or transferred without vascular repair with early survival dependent on absorption from the recipient bed while revascularization is taking place (unless specified as a vascularized graft, in which case it is the same as a flap only common usage does not refer to it as such, e.g., vascularized bone graft).
Graft versus host disease: GVHD, condition in which transplantation of immuno- competant cells into a foreign recipient, such as in bone marrow transplantation, results in immunologic attack of the recipient by these cells.
Haplotype: segment of genetic information usually transmitted as a unit and coding for a specific gene product.
Heterotopic: denotes the presence of an anatomic structure, organ or tissue(s) in a location other than where it is normally found.
Histocompatibility: comparative genetic identity of individuals, which if sufficiently similar permits successful allografting between them. Major and minor histocompatibility barriers refer to the degree of dissimilarity present between donor and recipient.
HLA: human leucocyte antigens.
HT: hand transplant.
Hyperacute rejection: rapid rejection (minutes to days) in some cases due to previously formed antibodies and characterized by rapid vascular destruction seen in primed recipients transplanted across major histocompatibility barriers. In the slower form, cell mediated immunity is implicated.
i.m.: intramuscular.
Inbreeding: mating of related animals over multiple generations in order to produce a genetically homogeneous population.
Induced or immune tolerance: condition characterized by a lack of rejection of a foreign anatomic structure, organ or tissue(s).
Interdigital pads: three pads of skin and thick subcutaneous tissue in the distal palm of Papio hamadryas anubis overlying the metacarpophalangeal joints of the digits excluding the thumb.
Internal fixation: hardware placed within the skin envelope permitting stable bony immobilization.
IU: international unit(s).
i.v.: intravenous.
kg: kilogram(s).
Lymphokine: non-specific helper factor produced by some cells involved in the immune response.

Lyophilization: freeze drying.
mg: milligram(s).
MHC: major histocompatibility complex.
mL: milliliter(s).
MLC: mixed leucocyte culture.
mm: millimeter(s).
Neurolysis: splitting a nerve's connective tissue sheaths longitudinally without transecting any axons.
ng: nanogram(s), billionth(s) of a gram.
NVFF: neurovascular free flap.
NVIF: neurovascular island flap.
Orthotopic: denotes the presence of an anatomic structure, organ or tissue(s) in a location where it is normally found.
Parabiosis: fusion of the vascular systems of two individuals.
Passive enhancement: situation in which short-term treatment against rejection leads to a condition of long-term or perpetual immunologic tolerance.
Phylogenic scale: evolutionary development organization of the species.
p.o.: per os, oral.
Primed recipient: a recipient having already been exposed to a specific set of foreign antigens.
r: (probably) rad.
Recipient: individual receiving the transplanted anatomic structure, organ or tissue(s).
RIA: radioimmunoassay.
s.c.: subcutaneous.
Second set rejection: rejection of an organ or tissue in a recipient having already been exposed to the specific set of foreign antigens (primed recipient). In some cases the period of time elapsed between exposures modifies the immune reaction at the second exposure.
Sensitization specificity: immunologic stimulation and preparedness to respond to a specific set of foreign antigens.
Sensory nerve distribution: area of skin supplied by a specific sensory nerve.
Serum trough level(s): serum drug levels in this case, taken at their lowest level immediately before the next dose of the drug is given.
'Switch' transplant: where each of a pair of individuals is both donor and recipient of the anatomic structure, organ or tissue(s) transplanted.
Syngeneic: genetically identical.
T-cell: T-lymphocyte.
Tenodesis: passive motion at a joint due to motion of an adjacent joint and usually in the opposite direction.
T-lymphocyte: thymocyte-derived lymphocyte.
TNVFF: transplanted neurovascular free flap.

Transplantation: transferring an anatomic structure, organ or tissue(s) from one individual to another usually genetically different individual (not used when transferring from one area of the body to another in the same individual).
μm: micron(s), micrometer(s), millionth(s) of a meter.
μmol: micromole(s), millionth(s) of a mole.
Unprimed recipient: (see first set rejection).
Xenograft: same as an autograft only transplanted to a recipient of a different species.

INTRODUCTION

The primary goal of reconstructive surgery is restoration of function and quality of life to patients with congenital or acquired deformities. Referrals from every surgical sub-specialty challenge the reconstructive surgeon. For their treatment, numerous ingenious operative procedures utilizing skin grafts and skin flaps, as well as grafts of bone, muscle, and nerve have been developed. Nevertheless, limitations remain in cases where there is insufficient or inadequate donor tissue for the required surgery. Thus, young adults with non-replantable upper extremity amputations can only be offered insensate prostheses and those with severe hand burns, although healed using skin grafts, frequently develop devastating function-limiting contractures. Unfortunately all too many patients must live with serious morbidity due to crippling and mutilation of appearance. For these situations, when the defect cannot be corrected with the individual's own tissue, transplantation of comparable tissue from donors would provide a possible solution.

In this context, a legend fascinates surgeons to this day. Twin brothers, Cosmas and Damian, the former a physician, the latter a surgeon, lived in the 3rd-Century AD devoting their lives to the sick, accepting no fee. Their charitable work came to an abrupt end with their martyrdom

near the turn of the century. At the Basilica which bears their names, built over two and a half centuries later in Rome, they apparently reappeared. According to legend, they came to perform the miracle of the black leg. The writings profess that the brothers removed the diseased limb of a devout member of the church and replaced it by transplanting the leg of a recently deceased Ethiopian Moor, hence the black leg (11, 25, 91, 126, 193).

Although modern plastic surgeons are far from being able to duplicate such a wondrous feat, the discovery and development in the early 1970's of a new drug, Cyclosporin A (hereafter CyA), has permitted dramatic breakthroughs in immunosuppression during transplantation. It has been found effective clinically and experimentally in the transplantation of a greater number of organs than ever possible using previous methods (24, 26, 27, 28, 29, 30, 37, 52, 73, 77, 78, 87, 89, 100, 101, 102, 110, 119, 121, 122, 127, 131, 134, 157, 165, 175, 177, 179, 180, 181, 182, 190, 191, 195, 204, 208, 214, 215, 216, 217, 230, 244, 246, 247). In the laboratory, CyA's capabilities have further amazed researchers in its successful use for the transplantation of numerous tissues (51, 132, 206) including skin (17, 50, 99, 100, 141, 142, 165, 235, 244, 246), muscle (10, 103, 241), and nerve (4, 5, 252, 253). The superiority of CyA over other immunosuppressive regimes has made successful

transplants of tissue for reconstructive purposes a realistic goal.

The requirements made of certain types of reconstructive tissue transplants differ significantly from clinical transplants undertaken until now in that their reinnervation is essential. Unlike renal and heart transplants that perform independently of direct neural regulation, digits and hands must be innervated to be useful. Therefore, new experimental data are required for the evaluation of reinnervation of such transplanted tissues in the presence of CyA before clinical application can ever be considered. To provide the possibility for such a study in a higher species, successful experimental models must be developed.

The primary objective of this thesis was therefore to design two primate models of transplantation for reconstructive purposes exhibiting long term survival. Prolonged survival was paramount to allow sufficient time for reinnervation to occur even if it were retarded by the process of transplantation or immunosuppression. One model was intended to allow future study of sensory reinnervation, the other to permit eventual assessment of motor reinnervation. Together they would ultimately provide the opportunity to study the effects of transplantation on skin, muscle, tendon, nerve, and bone.

BACKGROUND

Overview of Transplantation in Reconstructive Surgery

Transplantation surgery derives its roots from the field of reconstructive surgery with its use of grafts and need for additional donor sources (7, 47, 105, 183, 186). Reconstructive surgery, in turn, was born of the necessity to close open wounds and developed with the discovery that tissue could be transferred from one area of the body to restore another as a graft or flap. The first written record of a reconstructive procedure dates back to India around the 6th-Century BC in which Sushruta describes the reconstruction of an amputated nose with the transfer of a forehead flap (7, 45, 46). In the beginning of the 1800's, Baronio published the first recorded experimental skin grafts on sheep thus proving that skin could be completely detached from the body during transfer (7, 25, 45, 105). By 1823, Bünger had described the first documented clinical skin graft when he transferred skin from the thigh to cover the nose of a patient (7, 45). These physical transfers of tissue within the same individual, or autografts, were a major step in providing a source for the restoration of both form and function.

In 1863, Bert reported his study of autografts (taken from and applied to the same animal), allografts (taken from an animal and applied to another genetically dissimilar

animal of the same species) and xenografts (taken from an animal and applied to another of a different species). He noticed different degrees of success for each type of skin graft (7, 205). These empiric observations became the foundation for a new discipline, transplantation medicine, and the first of an increasing number of studies. In 1903, Jensen was the first to suggest that the immune response was the mediator of graft rejection (7, 105, 205), and by 1924 Holman not only described the difference between the rate of rejection of first and second set skin grafts, but also recognized sensitization specificity (7, 118, 205).

World War II, with its numerous casualties and burn victims, magnified the need for a suitable and inexhaustible source of skin for wound coverage. Interest in the problems of skin allograft rejection was renewed. The fateful meeting between Medawar, an English zoologist, and Converse, an American plastic surgeon, during the early part of the war, began a new chapter in the history of transplantation (7, 47, 205). Medawar, after this encounter, began working with an English plastic surgeon, Gibson, in studying skin allografts (25, 49, 105, 163, 164, 205). His subsequent lifelong quest in the laboratory to understand the process of rejection and induced tolerance later won him the Nobel Prize for Medicine (7, 47, 105, 163, 164, 205). Prompted by his meeting in England with Medawar and his experience in treating combat casualties, Converse, upon returning home,

became determined to unlock the mysteries of human skin allografting. He received one of the first grants for transplantation research in 1950 (47) and later proposed the first international meeting on transplantation which took place in 1954 (47, 205). With his associates, using a systematic program of experimental skin grafts, he went on to document human skin allograft behavior (47, 48, 184, 185, 186, 187, 188, 205). One of his colleagues, Rapaport, continuing this work in collaboration with Dausset, helped clarify some basic principles in immunology (47, 69, 70, 71, 72, 186, 187). These contributed clues toward elucidating the significance of histocompatibility in the outcome of allografting and eventually clarifying the role of the human leucocycte antigens (hereafter HLA) in clinical organ transplantation (47, 105, 164, 186, 205). By June 1958, it was announced that the new specialty would be publishing the Transplantation Bulletin within the pages of the Journal of Plastic and Reconstructive Surgery (236).

These contributions of reconstructive surgery to transplantation medicine laid the ground work for its clinical application. Although attempted sporadically before, in the early 1950's, several groups undertook kidney transplants (105). In 1955, Hume reported attempting nine such transplants using cadaver material (25, 47, 105, 207). Steroids, a product of the war effort (105), were used in some of these patients but only one patient survived to

around 6 months. The successful transplantation of a kidney between identical twins by Joseph Murray, a plastic surgeon at the Peter Brent Brigham Hospital in Boston, published in 1955, demonstrated that transplantation was technically feasible (167). He subsequently repeated the feat between several other twin pairs (25, 105). Therefore, its wider application awaited only the development of immune modulation that would reliably prevent rejection. Although total body irradiation in 1958 had permitted limited success (25, 105, 164), a major step was taken when the immunosuppressant properties of 6-mercaptopurine were noted in the late 1950's (24, 27, 164, 200). Within a few years its analogue, azathioprine, underwent clinical trial in kidney transplantation (163, 164, 168). Prednisone was soon added to azathioprine in order to achieve an additive effect of two drugs with different modes of action (105, 164, 170).

Despite the many modalities that have been tried over the past twenty years to improve graft survival and to increase the number of transplantable organs, the combination of azathioprine and prednisone remains the mainstay of standard immunosuppressive therapy to which all other regimes are compared (25, 28, 37, 105, 203, 243). A single more recent discovery, CyA, stands alone in the field of immunosuppression as a major advance. With the advent of this more potent immunosuppressant, the history of transplantation may travel full circle. Reconstructive

surgery, which contributed to the development of transplantation with its quest for additional donor sources, may in turn be advanced by this new transplant drug. Should CyA permit the successful transplantation of skin and other tissues previously found to be impossible utilizing other methods, a new chapter in the history of reconstructive surgery may be written.

1. Cyclosporin A

In 1969-70, two new strains of fungi imperfecti were discovered by the Microbiology Department of Sandoz[1] on routine study of soil samples brought from the United States of America and Norway (14). A two component metabolite mixture isolated from the fungal extract showed evidence of weak anti-fungal activity, very low toxicity and, after further study, a strong immunosuppressant potential (12, 15, 24, 114). The purification of CyA from this mixture in 1973 allowed the detailed evaluation of this compound and its surprising characteristics (14). Subsequently, its structure was elucidated and the molecule was successfully synthesized in 1980 for large scale production (14, 242).

In 1977, the first publications appeared documenting this drug's effectiveness in experimental laboratory transplantation (27, 134). The impressive results for heterotopic heart transplantation in the rat and renal

[1] Sandoz, Ltd., CH-4002, Basle, Switzerland

allografts in dogs stimulated widespread interest in the drug. Soon after, clinical cadaver kidney transplantation (30) as well as treatment and prevention of graft versus host disease during bone marrow transplantation (179, 180) were undertaken with the use of CyA. Since then, it has been found clinically effective in kidney (24, 26, 28, 29, 30, 37, 87, 89, 127, 131, 157, 165, 204, 215, 216, 230), heart (37, 102, 131, 175, 177, 190, 191, 217), lung (30, 190), liver (26, 28, 131, 165, 217), pancreas (37, 157, 195), and bone marrow (37, 110, 121, 165, 179, 180, 181, 182, 214) transplantation. Experimentally, in the laboratory, it has allowed transplantation of other organs (52) and tissues (51, 132, 206). Most significantly, CyA has permitted experimental skin (17, 50, 99, 100, 141, 142, 165, 235, 244, 246), nerve (4, 5, 252, 253), and muscle (10, 03, 241) allcgrafting as well as limb transplantation (10, , 92, 93, 115, 129) in various animal models previously found to be disappointing in most cases when using other forms of immunosuppression (76, 96, 117, 136, 178, 222, 235, 250).

The molecular mode of action of this lipid-soluble cyclic undecapeptide (11 amino acids) is to a great extent still elusive. The normal method through which the body coordinates the allogenic graft rejection response is a complex repertoire of primary and substitute mechanisms

dependent on the type of antigen and its route of stimulation (1).

Although the immune apparatus consists of a varied group of specialized cells (T-lymphocytes, B-lymphocytes, monocytes / macrophages), and interacts with other physiologic non-specific pathways (e.g., the complement-clotting mechanism) and cellular mediators (e.g., polymorphonuclear leukocytes) (1), it is generally agreed that the T-lymphocyte is pivotal in the rejection process (38). T-cells are themselves subdivided not only by antigen recognition specificity, but by their roles. Some serve in a regulatory function of the immune response (e.g., helper T-cells, suppressor T-cells), while others work in an effector capacity (e.g., production of lymphokines, cytotoxic killer T-cells) (1). Although the part played in the rejection process of many T-cell subtypes is unclear, cytotoxic T-cells and helper T-cells have been shown to be involved.

Specialized cells derived from the monocyte/macrophage lineage (e.g., dendritic cells, Langerhans' cells) present antigens to the immunologically prepared helper T-cells and produce interleukin 1 (1). The helper T-cells respond by proliferating, some stimulating B-cells into proliferation and antibody production, others producing lymphokines (non-specific helper factors) (1). The inflammatory process and interleukin 2 production are quickly engaged (1).

Interleukin 2 further stimulates all T-cells to proliferate and specifically activates cytotoxic killer T-cells which lyse the foreign cells recognized by their specific antigen (1).

Although several sets of antigens differentiate human individuals from each other (including the ABO blood group system and sex associated H-Y antigen), the HLA is the strongest of the transplantation antigens (1, 104) and represents the major histocompatibility complex (hereafter MHC).

The MHC is a small segment of genetic material on the short arm of autosome number 6 (18, 104). Its gene loci A, B, C, and D, determine cell surface antigens and are linked so that they tend to be transmitted as a genetic unit called a haplotype. One haplotype is contributed by each parent. Each locus within the haplotype, although only capable of coding for the synthesis of one antigen, has many potential alleles in the population as a whole.

The HLA is subdivided into Class I and Class II antigens. The former were originally identified by serologic methods and include HLA-A,-B, and -CW (18). Class I antigens are found in virtually all nucleated cells and in body fluids (18). They activate and interact with cytotoxic T-cells stimulating foreign cell lysis and activate the antibody response (18). HLA-D antigens are Class II, first identified by mixed leukocyte culture (hereafter MLC)

proliferation and are subdivided into D_R (also written HLA-DR), D_Q, and D_P (18). These antigens are more limited in their distribution, concentrated mostly in key locations for the purpose of setting the immune response in motion (e.g., B-lymphocytes, antigen presenting cells such as those from monocyte/macrophage lineage, Langerhans' cells, dendritic cells, activated T-lymphocytes, epithelial cells of various organs, bone marrow precursor cells, some neoplasms, capillary and glomerular vascular endothelial cells) (18, 88, 218, 233). Class II antigens activate helper T-lymphocytes, stimulate the MLC and can also evoke antibody response (18). Thus Class II antigens stimulate the afferent limb of the immune response (i.e., recognition and proliferation) while Class I antigens attract cytotoxic T-cells which are responsible for the efferent process of cell destruction (18).

In humans, the D locus (Class II) antigens seem to play an important role in transplantation histocompatibility because of their effects on T-cell stimulation (233). HLA-DR matching influences significantly the prognosis of allografted tissues (234), particularly certain DR specificities which may contribute to a more intense immune response (38) making DR matching of particular value (130). On the other hand, in previously sensitized recipients, matching of the A and B loci (Class I) antigens may be of greater importance than matching for the D locus to avoid

hyperacute rejection (233). Although the importance of HLA typing in transplantation is still being evaluated, there does seem to be favorable effect on long-term results with HLA-A, -B, and -DR typing particularly in patients treated with other than CyA immunosuppression (38). Antigens of the C locus at present appear much less important than the others. With HLA loci incompatibility, the rejection of an allograft by cellular immunity usually occurs within 10 days for an unprimed recipient (first set rejection) (1, 48, 158, 185, 186, 188) and within 4 to 6 days for a primed recipient (47, 158, 185, 186, 188); rejection being indicated by mononuclear cell, lymphocyte, and macrophage infiltration.

ABO blood group matching is clearly essential in transplantation to prevent hyperacute rejection when immunologic manipulation is not used (39, 130). There is also a newly-discovered Class I-like antigen system present on the vascular endothelial cells whose importance is becoming increasingly well understood (38, 39, 40). This system seems to be the most consistently involved in patients who reject an allograft and is becoming essential in the pre-transplant evaluation of potential recipients.

Although the role played by CyA in immunosuppression is unclear, there is unanimous agreement that the ability of CyA to inhibit predominantly T-lymphocyte-dependent immune responses is the factor inducing tolerance of transplanted tissue across major histocompatibility barriers in a variety

of animal models(13, 22, 31, 37, 113, 114, 165, 170, 245). In vitro studies indicate that CyA has a differential effect among T-lymphocyte subpopulations permitting the induction of suppressor T-lymphocytes while inhibiting the activation of cytotoxic T-cells (13, 22, 31, 37, 38, 113, 114, 139, 165, 170, 233). Although it appears that the latter are primed and recognize the foreign antigen, the immune response is prevented from progressing any further by CyA's inhibition of interleukin 2 production by helper T-cells (22, 31, 37, 38, 127, 170, 240), and apparently, interference with the precursor cytotoxic T-cell's ability to respond to this proliferative trigger (37, 38, 137, 165, 170, 176). Cyclosporin A can also control the expression of Class I antigens and even prevent Class II antigenic expression on endothelial cells (38). This drug therefore appears to effect both the efferent and afferent limbs of the immune response to achieve immunosuppression.

2. Skin transplantation

Skin, as the major barrier between individual and environment, has been found not only to be more immunogenic than the kidney (17, 196) or nerve (146), for example, but to undergo rejection more readily than other organs (17, 40, 196, 246) or the other tissues present in a limb (136, 146). Although Langerhans' cells are believed to be essential in provoking allo-agression by the recipient immune system due

to their Class II antigens (53), all three cell lines of the epidermis (Langerhans' cells, keratinocytes, and melanocytes) seem to stimulate lymphocytes (196). This observation may explain the greater immunosuppression dosage requirements for skin allograft survival as compared to that for certain solid organs (2, 246). Skin grafts survive less than 3 weeks before rejection even under good histocompatibility matching in several species (17, 23, 41, 50, 99, 136, 141, 142, 146, 158, 219, 235, 246).

Although various immunosuppressant regimes have been tried in the past, prolonged skin allograft survival has been difficult to achieve. Despite numerous animal models, treatment with steroids, cyclophosphamide, 6-mercaptopurine, antilymphocyte plasma, methotrexate, azathioprine, and azaserine, some in combination, others alone, have had only modest success (235). Some drugs were entirely ineffective and others improved graft survival only slightly (in a single case up to 37 days), but most harboured unacceptable toxicity (235). With antilymphocyte serum, somewhat longer periods of allograft viability were attained. In one study with high doses administered, healthy grafts were present at 41-56 days post-transplant but the animals died of toxicity (235). When used for a restricted period of time followed by steroid maintenance, anti-lymphocyte serum could sustain some grafts for as long as 49 days and in three isolated cases to beyond 100 days (235), but it was not until the

development of CyA that research into skin transplantation was transformed.

With this new drug administered over a wide range of dosages (from a maintenance dose of 8 mg/kg s.c. every 4 days to 150 mg/kg/day p.o.) in various species, skin allografts have been shown to survive in many cases at least as long as the drug is continued (17, 50, 99, 100, 141, 142, 165, 235, 244, 246). After cessation of adequate CyA therapy, graft survival ranges, on the average, were from 3-23 days beyond the end of treatment as compared to controls in which total survival was only 8-15 days (17, 50, 99, 141, 142, 235, 246). Most published studies dealing with skin transplantation under CyA immunosuppression have utilized skin grafts (17, 50, 99, 100, 141, 142, 235, 244, 246). Skin flaps on the other hand had never been studied in this manner with CyA prior to this project and there is very little information about any other modes of immune modulation for such flaps (198). With the use of this drug the relatively greater immunogenicity of skin may no longer remain an obstacle for future investigations.

3. Nerve transplantation

Nerve tissue allografts do not appear to be as immunologically challenging as skin allografts (146). Although rejection does occur (65, 117, 146, 222, 250, 253), nerve fibers have been shown to successfully regenerate across

fresh foreign grafts in the non-immunosuppressed rat (65, 149, 254). Nevertheless, there seems to be some disagreement as to the relative importance of major versus minor antigenic mismatching and the significance of allograft length in these cases.

In more practical terms, the goal of achieving consistently good results for longer segments of nerve allograft, as would most commonly be required in the clinical setting, has been sought utilizing various techniques. Irradiation, pre-degeneration, freezing, lyophilization and combinations thereof as well as other techniques have been tried in pre-treating allografts in view of decreasing their antigenicity (65, 147, 148, 254). Although pre-treatment offers the theoretical advantages of limitless supply without the need for altering host defences, reports in the literature of such studies have provided conflicting results (65, 147, 148). Pre-treatment, in addition, does not offer a direct solution for the transplantation of nerves as an integral part of a composite tissue transplant, a hand for example.

Immunosuppression, on the other hand, has shown some promise. Immunologic tolerance at birth and "radical immunosuppression" (using thymectomy, total body irradiation, and bone marrow reconstitution), although impractical in routine clinical practice, have allowed allografting across minor and major histocompatibility

barriers in the rat (146, 254). Nerve regeneration through 4 cm allografts in the immunologically tolerant animals was successful as compared to controls in which reinnervation of target muscle did not occur (254). Radically immunosuppressed rodents, equally showed no immunologic evidence of sensitization when tested 20 days after grafting (146).

Pharmacologic immunosuppression, clinically more useful, has also been assessed in rat nerve allografting across major antigenic barriers. Although azathioprine and hydrocortisone alone or in combination have been shown to improve axon regeneration through segments of allograft over that in untreated controls when measured by electrophysiologic and histologic parameters, results did not compare to those of autografted rats (147).

From the experiments of Zalewski and Gulati, Schwann cells in peroneal nerve grafts and vagal nodose ganglia have been shown to survive in experimental animals (252) suggesting the possibility that they could support regenerating axons. These investigators subsequently demonstrated that axons will grow through allografted peripheral nerves in rats treated with CyA (253). In addition, CyA has been shown to reduce or eliminate histologic evidence of rejection, depending on the dosage schedule used (4, 5, 252, 253). It can even prevent immunologic responsiveness to the graft when given at a low

non-toxic continuous daily dosage (5 mg/kg s.c.) (4, 5). Functional, electrophysiologic, and histologic evaluation of such allografted rats has shown them to be comparable to those with syngeneic grafts (5). Despite such encouraging results, short-term (28 days) treatment with CyA did not seem to provide long-term (88 days) survival of nerve allografts suggesting that its long-term use may be necessary (although repair of the distal end of each graft, permitting the possible influence of target muscle trophic factors, had not been undertaken in this study) (253).

Although the results with this drug have been impressive, all of these studies have dealt with segments of allografted nerve. None have addressed the important question of whether recipient nerve endings can recognize and interact with foreign donor motor end plates or sensory structures, the basis of reinnervation in reconstructive transplantation.

4. Muscle transplantation

Although muscle allografts have also been found to be immunologically less stressful than skin transplants (241), they are normally rejected by 10 days or after 2 weeks depending on the animal model used (103, 241). They have not generated as much interest as some of the other allografts, but have been studied with some success in the laboratory. Induction of immune tolerance at birth and in

the adult animal despite being impractical in humans, achieved survival of muscle transplants up to 200 days in some cases (241). Immunosuppression, a clinically more useful modality, with anti-lymphocyte serum three times a week has also been shown to be effective for up to 30 days after cessation of therapy (241).

Cyclosporin A has more recently been evaluated in muscle allografting. Watt et al transplanted minced muscle in mice between strains differing at the MHC locus (241). They found that a higher dose (200 mg/kg/day p.o.) of CyA was more effective than a lower one (150 mg/kg/day p.o.) in short-term treatment (20 days) and could maintain the graft for up to 12 days beyond discontinuation of therapy. Interestingly, the muscle surviving in the recipient consisted of a mosaic of donor and recipient cells, probably in part as a result of the form in which the cells were transplanted (minced). Gulati and Zalewski, transplanting an entire muscle in rats having both major and minor histocompatibility differences, used much lower CyA doses to avoid the toxicity seen by Watt's group (103). Despite receiving only 5 mg/kg/day s.c., grafts were not rejected as long as treatment was continued. Even though no neurovascular repairs had been undertaken, reinnervation occurred by anomalous routes, returning muscle bulk to about 50% of normal. Tests of function were not attempted so the efficacy of the newly-formed connections is unknown.

However, the fact that some of the muscle bulk returned suggests that there might have been a positive trophic effect exerted on the transplanted muscle. This data therefore suggests that nerves can not only regenerate and become remyelinated in the presence of CyA, but that donor muscle may be capable of receiving recipient nerves that provide the trophic signals necessary for its maintenance.

In a preliminary communication, Black et al did measure the contractile properties of muscles transplanted as part of a limb allograft in the rat (10). The transplants had been undertaken 274 and 701 days earlier respectively across a major immunologic barrier. The maximal tetanic contractions for the tested muscles were on the average 65% those of the normal side. Although the authors attributed this to "simple atrophy", no control CyA treated or untreated autograft values were mentioned. This information, despite becoming available well after the beginning of our study, provided tangible hope that neuromuscular interactions are possible between recipient and donor tissues in the presence of CyA.

5. Bone transplantation

Rejection of non-vascularized allografts occurs with bone as with other tissues (41, 54, 169), but much less often or not at all with articular cartilage (23, 95, 138). Under most conditions bone allografts can sensitize the host

sufficiently to induce earlier rejection of a skin graft transplanted from the same donor (23, 41, 251). The presence of marrow in some of these allografts stimulates a more easily detectable response than when it is washed away (23). Nevertheless, the discovery in vitro of what are known today as Class I transplantation antigens on the surface of the bone cells themselves (169) may contribute to the impairment of new bone formation around fresh marrow-free iliac bone grafts even when these do not stimulate premature rejection of a donor specific skin graft (23).

Pre-treatment such as boiling, freezing, and freeze drying of bone grafts can diminish their antigenicity (23, 91). While freeze dried grafts seem to retain a small degree of immune stimulation (169), freezing does not significantly alter long-term outcome of experimental allografts when compared to fresh ones (112). In both kinds of grafts late joint disintegration occurs. This is believed to be related to impeded or disrupted revascularization noted in allografts with resultant aseptic necrosis (95, 107, 112, 220, 251). Despite such negative experimental data, non-vascularized allo-transplantation of joints and parts thereof are used clinically (91, 239). Apparently good results can be achieved, particularly in cases where the bone is used for packing spaces or only one surface of the joint is replaced, but little detailed evaluation is provided in these papers.

Although non-vascularized bone and joint grafting offers the advantage of a shorter operative time, living bone would be preferable for the reconstruction of certain types of defects. Large defects, particularly in weight-bearing bones or in the presence of a poorly vascularized bed (151), especially in joints where degeneration occurs following failure of the vascular supply to the graft (95), successful vascularized bone grafts would be of clear benefit. Since the body does not include a dispensable source of large joints, vascularized joint transplantation has been studied experimentally. Without immunosuppression these transplants were readily rejected (189, 206, 251). Using azathioprine intermittently in the dog, survival for as long as 18 months in a non weight-bearing heterotopic location was apparently possible (189). In another study, orthotopic transplantation in the dog was achieved with patent anastomoses proven by angiography for up to one year in just over 20% of animals (95). This low success rate occurred despite combination therapy using anti-lymphocyte serum, azathioprine, and prednisolone, due to chronic rejection resulting in function inferior to those of control autografts. In another study in the rabbit, immunosuppression using azathioprine, methylprednisolone, and in some animals, irradiation, although increasing survival time to a small extent in the non-irradiated cases

as measured by bone scanning, did not do so in a statistically significant manner (251).

By contrast, in a letter to the editor by Siliski, CyA alone given to rabbits at 15 mg/kg/day for orthotopic transplantation of knee joints permitted survival of bone and cartilage for at least 3 months (when the grafts were harvested for study) in nearly 36% of cases (206). Although there was no mention of functional outcome, two grafts in immature animals did demonstrate ongoing growth at the epiphyseal plates. Cyclosporin A therefore may prove to be beneficial in the experimental transplantation of large segments of vascularized bone, particularly when these include joints and periarticular structures such as those of a hand for example.

6. Limb transplantation

Research into the transplantation of limbs began even before the advent of immunosuppressants (201) and prior to the advent of CyA results were generally disappointing (76, 96, 136, 143, 178). The few that were successful during this period utilized techniques inapplicable to humans. Schwind, for example, over a two week period gradually transferred the hind limb from an immunologically immature rat to another using parabiosis (201). He noted that only one third of such transplant pairs survived long enough to be successful, but that the percentage could be increased

using previous mutual donor-recipient neonatal transplantation of salivary or splenic tissue. Tweed also noted prolonged survival of allografted guinea pig hind limbs when previous donor-specific splenic allografts had been accepted (237). Lapchinsky, reportedly achieved immunologic tolerance in a dog by complete exchange transfusion from the donor when the recipient was 6 days old (159). Nine months later the limb was transplanted with survival noted at just under 2 months.

Attempts were also made to induce tolerance in adult recipients. Poole et al successfully prolonged rat limb allograft survival in recipients using a previous anti-serum-enhanced kidney allograft (178). It was noted that the longer the time elapsed between kidney and limb transplantation, the longer the limb survived. Curiously, all the rats in this experimental group died between 21 and 207 days after limb transplantation with no explanation given as to the cause of death. In the same study, the authors tried to induce non-reactivity in normal recipients with the use of allo-antibodies directed against antigens present on the donor lymphoid cells. All limb allografts in this group were rejected by 16 days.

Several other authors undertook adult animal limb transplantation using various combinations of immuno-suppressive drugs without the benefits of CyA. Goldwyn et al used 6-mercaptopurine in mongrel dogs (96). Treated dogs

showed a slightly prolonged hind limb allograft survival of 11 to 28 days compared to 6 days in untreated controls. Despite the minimal improvement, it was at the expense of fatal side effects (hemorrhagic pneumonitis, leukopenia, hepatocellular necrosis or suppuration). A similar result was described by Doi who attempted transplanting rat limbs with several drug regimes (76). Although treatment with azathioprine and prednisolone permitted some increase in allograft survival (4 out of 7 transplants survived longer than the average control of 12.5 days), all rats succumbed to side effects of immunosuppression before rejection could occur. None of the other treatment groups studied (6-mercaptopurine and prednisolone, azathioprine alone, prednisolone alone, and 6-mercaptopurine alone) attained significantly prolonged survival and 11 of these 14 rats with technically successful transplants also died of complications prior to rejection.

The only study to have reported some prolonged recipient and allografted limb survival prior to the advent of CyA was by Lance et al (136). Using various potent combinations of anti-lymphocyte serum, azathioprine, hydrocortisone acetate, thymectomy, splenectomy, exchange transfusion, and splenic cell suspension, transplantation of canine hind limbs was undertaken between unrelated registered beagles. Although significant prolongation of allograft survival was shown in dogs receiving continuous

immunosuppression (antilymphocyte serum or hydrocortisone acetate and azathioprine), all died or had to be terminated due to serious complications including pancytopenia, wound infection, systemic sepsis or self mutilation. Greater success occurred in three animals receiving a short course of massive immunosuppressive drug therapy, with or without splenectomy and/or thymectomy, and followed by induction of immune tolerance using donor splenic cells or exchange transfusion. Although these dogs had a somewhat unstable course, long-term survival was achieved with one rejecting on day 200 and the other two surviving beyond 60 and 300 days respectively.

In 1964, Gilbert and Panchana transplanted a human hand with part of the forearm from a cadaver to an injured sailor in Ecuador (160). Postoperatively, Wilson and Goldwyn from Boston were requested to help with the immunosuppression. Prednisone and 6-mercaptopurine were used, the latter replaced by azathioprine a few days after transplantation. Postoperative irradiation (150r) was administered. Beyond the initial report, no further information was published on the outcome of this clinical experiment.

The discovery of CyA quickly rekindled research in limb transplantation. In 1982, Black et al published the first data on adult rat limb transplants using CyA immunosuppression (11). In this preliminary study, four

Lewis recipients underwent transplantation of a hind limb from hybrid Brown Norway (BNxLew F1) rats. Cyclosporin A was administered at a dosage of 25 mg/kg/day s.c.. Mean survival of the allografts was an impressive 101±13 days in the treatment group compared to 18±5 days in the control group. One year later, the final results from this experiment became available (115). Of the five groups studied, the first two control groups underwent transplantation without treatment (group 1) or with the administration of the solvent (20% Tween 80 in anhydrous ethanol) alone (group 2). A donor specific blood transfusion (1 mL Brown Norway whole blood) was given 1 week prior to transplantation in groups 3 and 4 with additional passive enhancement (1 mL Lewis anti-Brown Norway allo-ascitic fluid) on the day of transplantation as well as 2 and 3 days later in group 4. Group 5 received 20 days of CyA at 25mg/kg/day s.c. post-transplantation. Rejection was defined as a 10°F fall in temperature of the allografted limb along with the earliest visual changes.

Although donor specific blood transfusion can prolong rat kidney allograft survival across even stronger histo-compatibility barriers (115), it did not succeed in doing so for the transplanted limbs (group 3), even when augmented with immunologic enhancement (group 4). In contrast, CyA produced a dramatically increased survival (group 5) as compared to controls (group 1) or solvent treated animals

(group 2). The mean allograft survival times in CyA treated rats were approximately 10.5 times greater than either group 1 or 2. There was no significant difference between these latter two groups. The longest successful CyA treated animal had not rejected its allograft at the time of publication 225 days after transplantation despite having stopped the immunosuppressant 205 days previously.

Fritz et al also undertook adult rat limb transplantation with success (92,93). ACI strain hindlimbs were heterotopically transplanted to the back of Lewis rats representing "a very strong antigenic mismatch". Four groups of five animals each were studied. Rejection was determined clinically by swelling, erythema, and skin changes, histologically by cellular infiltrate, skin atrophy, and vasculitis, and immunologically by the presence of cytotoxic and hemaglutination antibodies. All controls (group 1) being administered the plain solvent (Miglyol 812) showed evidence of clinical rejection within 14 to 20 days. Amongst the CyA treated animals receiving 10 mg/kg/day s.c. for a duration of 7 days (group 2), 21 days (group 3), or continuously (group 4), the results were significantly better. Only in the short-term group (group 2) did 2 out of 5 animals show clinical signs of rejection similar to those of the controls (group 1) 4 to 5 weeks after discontinuation of therapy. No treated animals developed strong histologic signs of rejection, and nine of these (3

from group 2, 1 from group 3, 5 from group 4) had no microscopic evidence of rejection at all. In contrast, all of the control rats (group 1) developed strong histologic evidence of rejection. Similarly, antibody testing revealed a strongly positive response in all untreated animals (group 1), as opposed to none in the continuous treatment group (group 4).

Kim et al also published a similar study in which adult rat limbs were transplanted orthotopically across defined histocompatibility barriers with varying regimes of treatment (129). Of six groups, the first (group 1) underwent limb replantation and the second (group 2) transplantation, both without immunosuppression. The next two groups received 10 mg/kg/day i.m. CyA, one for a period of 2 weeks (group 3) and the other for 2 months (group 4). The remaining two groups received 10 mg/kg/day of continuous intraperitoneal azathioprine (group 5) and prednisolone (group 6) respectively. The replanted limbs (group 1) showed some edema during the first week and denervation atrophy, but otherwise had a normal long-term appearance. In the control group (group 2), clinical signs of rejection appeared on the average at 6 days. The short-term CyA rats (group 3) showed some edema during the first 7 to 10 days followed by normal appearance until on the average 32.9 days following transplantation (18.9±3.0 days beyond the end of treatment). In the long-term CyA group (group 4),

appearance of the limbs was similar to the replants until a mean of 67.7 days (7.7±1.9 days past the end of CyA administration), when signs of rejection appeared. This result was in contrast with the azathioprine (group 5) and prednisolone (group 6) groups in which rejection appeared on the average at 7.2 and 7.8 days respectively during continuous therapy. These studies have therefore become the standard to which all subsequent studies are compared.

Overview of Microsurgery

The surgical use of the microscope began in 1921 for drainage of a middle ear infection (56). The otorhinolaryngologists soon adopted the new instrument for a variety of operations in the middle ear (56, 57). In 1953, Carl Zeiss introduced the modern operating microscope with coaxial illumination and variable magnification (56). Before long the ophthalmologists discovered its advantages for repair and reconstruction in the confines of the eye (56, 57). However, it was not until 1960 when Jacobson and Suarez reported the use of the microscope for the reliable anastomoses of vessels less than 2 mm in diameter (124, 125) that plastic and reconstructive surgeons took particular note of this increasingly popular tool (57). Vessels of decreasing size could thereafter be repaired with increasing patency rates paving the way for replantation surgery and free tissue transfers (227).

Reconstructive microsurgery quickly became an expanding specialty. The introduction of microsurgical technique (43, 62), improved instrumentation, and most importantly, the continued development of the operating microscope provided the necessary framework for this new discipline. Instruments, clamps, and suture material quickly evolved to meet the requirements of this demanding type of surgery (43, 60, 61, 94).

Soon reports of both experimental amputation and replantation in laboratory animals (19, 20, 21) and successful salvage of accidentally amputated human extremities (120, 150) at progressively more distal levels with increasingly small vessel diameters (43, 57, 63, 82, 94, 227) including digits (133) began to appear in the literature. From these reports, the possibility that large segments of tissue with their own blood supply could be transferred from one area of the body to another in one stage deserved consideration. While experimental work with free vascularized tissue transfers continued in the laboratory (20, 64, 97, 135, 174, 224), the critical step of sacrificing tissue from a healthy area of the body to reconstruct a deformity, entirely dependent on microsurgically repaired vessels, was achieved in humans (64, 83, 84, 85, 94, 202, 227). Intestine (43, 202), the toe (44), omentum (156), skin (59, 108, 173), and bone (226) were all successfully transferred as free flaps (or free

vascularized grafts). The door was open for all forms of free tissue transfer limited only by the surgeon's imagination and the painstaking determination of blood supply (55, 154).

With the early work of Smith, who saw the potential for the operating microscope in peripheral nerve surgery (210), previously dismal results could begin to improve with the birth of microsurgical nerve repair (65). The possibility of restoring sensation and even muscle function multiplied the scope of reconstructive microsurgery (109, 224). Nerve repairs became more sophisticated (98, 249) and the indications for the use of nerve grafts were clarified (161, 162). Even vascularized nerve segments, after having been developed in the laboratory animal, have been tried clinically (225). These were designed to improve the outcome in cases of wide nerve gaps where either a large caliber graft is required or to span a severely scarred bed.

Soon after publication of the first clinical free toe to hand transfer (44), reports multiplied as the foot became a source for the replacement of amputated digits without adding to the morbidity of the other fingers (83, 144). This method proved to be particularly useful for thumb replacement in severely traumatized hands with vascular damage preventing pollicization (144). While providing a new "thumb", this technique has allowed mastery of manipulation and sensory cortical integration to occur

without difficulty since the tendons and nerves to the toe are anastomosed to the stumps of those to the missing thumb (152).

Neurovascular free flaps were soon developed to resurface insensate areas of the body of particular functional importance such as the hand (66, 67, 68, 166). Muscle flaps, transferred with microsurgical repair of their vascular pedicle as well as their motor nerve, next provided animation in facial paralysis (109) and finger motion in forearm injuries (123, 153).

Since the 1960s, microsurgery has grown into the refined surgical technique it is today. Few surgical specialties have not, in some way, been affected by its development. Reconstructive microsurgery has provided an armamentarium of free tissue transfers in a multitude of types, shapes, and sizes, some providing coverage (e.g., skin) or bulk (e.g., omentum), others achieving movement (e.g., innervated muscle) or sensation (e.g., nerve), and still others permitting return of structural integrity (e.g., bone) or anatomic functional continuity (e.g., intestine).

It is now possible to expand the reconstructive horizons further and push the common frontier between transplantation and reconstructive surgery one step ahead. The time has come to develop models in which it will be possible to study the survival of transplanted flaps and

functional biomechanical units as an integral part of the interface between an individual and the environment.

OBJECTIVES AND RESEARCH PLAN

Cyclosporin A has been shown to significantly prolong survival of whole limb transplants in the adult rat (10, 11, 92, 93, 115, 129). Nevertheless, the question remains as to whether transplantation between inbred strains of rats poses a sufficient immunologic challenge for allograft survival since rats have been shown to express passive enhancement with relative ease (1), despite some strain variation, in contrast to the primate or dog (88). Examples include prolonged cardiac transplant survival following a short course of medication (165, 244, 246, 247), even to greater than 100 days after receiving only 15 mg/kg/day i.m. of CyA for 7 days (122) and kidney grafts lasting <u>indefinitely</u> after 1 week of daily doses or 5 doses of alternate day oral CyA therapy (119, 165, 208, 244). In the rabbit and some other animal models, although the time periods are generally less impressive, prolonged survival has also been noted (24, 77, 78, 101, 165).

One theory for the explanation of this phenomenon in the rat kidney, which if correct may apply to other organs and tissues, and perhaps even to some other animals, is that proposed by Fabre (88). It suggests that the rat may lack Ia antigens (now known as Class II or HLA-D) on the vascular endothelial cells in the kidney in contrast to the situation in humans. Cells with this antigen, as noted previously,

are particularly important in stimulating the afferent limb of the immune response by antigen presentation (88, 218). While present only on interstitial dendritic cells in the rat kidney, these Ia (Class II) antigen positive mobile cells may soon be replaced by ones of recipient origin. This phenomenon may explain the short-term need for immunosuppression in the rat. The human vascular endothelium, on the other hand, may not ever be able to rid itself of these antigens since they are found on the immobile endothelial cells themselves. Interestingly, rejection often appears to involve a significant vascular component, whether manifested by direct endothelial cell injury (such as in acute renal rejection, primarily a T-lymphocyte mediated occurrence) or actual luminal occlusion (such as in hyperacute renal rejection, an antibody dependant process in previously sensitized patients) (1, 251).

It therefore is questionable whether findings in the rat can be extended to humans or non-human primates (88). The rat limb transplantation studies have nevertheless become the cornerstone for further research. They have also provided an impetus to answer the critical question: can limbs or portions thereof be transplanted successfully between entirely unrelated individuals of a primate species?

Additionally, tissue transplants for reconstructive purposes, as noted earlier, differ markedly from previous

clinical transplants in that their reinnervation is essential. Unlike renal and heart transplants that maintain function independently of direct neural regulation, digits and hands must be innervated in order to be useful. Experimental data are therefore required evaluating reinnervation of such transplanted tissues in the presence of CyA before clinical application can ever be considered.

In order to provide such data, new experimental models with long-term survival must be designed. Therefore, the aim of this project was to design primate transplant models and to successfully achieve sufficiently long survival for functional motor and sensory reinnervation to have occurred in the transplanted tissue by recipient nerves under CyA immunosuppression.

The experiments were divided into three stages: **Stage I:** Design of a neurovascular free flap (hereafter NVFF) and a hand transplant (hereafter HT) model in the primate. **Stage II:** Gain experience with Cy A immunosuppression. **Stage III:** Undertake transplantation of the TNVFF and HT models in the primate.

MATERIALS AND METHODS

Despite detailed protocols resulting from extensive search of the literature and consultation with various specialists, constant improvements were implemented throughout the course of this transplantation project. Unless stated otherwise, the information provided in this section is the most updated procedure felt to achieve the best results. Most of the modifications were obtained through trial and error in the course of the study.

Experimental Animal

The baboon (Genus papio) was chosen as the experimental animal on the basis of several important features including the following: 1) similarity in anatomy of the hand to that of humans, 2) similarity of function of the transplanted tissues to that in humans, 3) possibility of incorporating into each model a tactile glabrous skin component, 4) comparability of sensory receptors, motor end plates and nerve pathways to those of humans, 5) adequate size of neurovascular structures in the adult for dependable microsurgical anastomosis and future neurophysiologic nerve recordings, 6) availability of previously established techniques, protocols, and standards for assessing return of neural function after peripheral nerve repair in this species (79, 229), 7) diameter of forearm bones permitting

solid internal fixation, 8) documented successful use of CyA during transplantation research in several primate species, 9) species closer to humans on the phylogenic scale, and 10) availability of a reliable supplier.

The animals were provided by a recognized primate dealer.[2] They were all wild young adult Papio hamadryas anubis originally from Kenya. Females were chosen for their smaller adult size and reportedly less aggressive behavior.

General Animal Care

Upon arrival to the supplier, they were subjected to a routine animal husbandry protocol (APPENDIX I). Each baboon was delivered to one of two primate care facilities utilized for this study[3] with an accompanying Veterinary Health Certificate (APPENDIX I).

Long-term animals were isolated from those in which all experimentation was completed within 2 weeks. Individuals were housed separately in squeeze-back primate cages according to the guidelines set by the Canadian Council on Animal Care (32). All animals were weighed on arrival and were within the 9-13.9 kg category requested from the supplier.

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[2]Charles River Research Primates Corp., P.O. Box 416, Port Washington, New York, 11050, U.S.A.

[3]Montreal Neurological Institute, Animal Facility Room 830, 3801 University St., Montreal, Quebec, Canada, H3A 2B4; McIntyre Medical Sciences Bldg., Animal Centre, 3655 Drummond St., Montreal, Quebec, Canada, H3G 1X6

Alimentation consisted of water ad libitum and a standard monkey chow diet supplemented with multiple vitamins, Ensure[4] liquid hyperalimentation and ferrous sulphate as required. Fruit and vegetables were given in reward following unpleasant manipulations. Yogurt served as the primary means for the administration of certain oral medications.

Tissue Typing

Since very few laboratories are involved in tissue typing Papio hamadryas anubis, it took considerable time to locate someone with whom to collaborate.[5] Six of the animals were tissue typed (#16,#17,#18,#19,#20,#21) using a similar technique (228) to that for human typing (171, 209). Tests showed that donor-recipient pairs #16-20, #18-19, and #18-21 had 2 chromosomal differences. Pair #16-17 shared one allele, but had a high probability of being different via further criteria. The rejection episodes seen in the remaining recipients (those not tissue typed), suggest that genetic dissimilarity existed in all donor-recipient pairs.

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[4]Ross Laboratories, Division of Abbott Laboratories, Ltd., 6300 Côte-de-Liesse Rd., Montreal, Quebec, Canada, H4T 1Z1

[5]R. Mark Sharp, Ph.D., Assistant Scientist, Southwest Foundation Biomedical Research, Dept. of Genetics, 7620 N.W. Loop 410, San Antonio, Texas, 78283, U.S.A.

Immunosuppression

In these experiments, CyA was routinely administered by intramuscular injections (73) beginning at around 22 to 23 mg/kg twice daily (100 mg dissolved in 810 mg Miglyol 812 and 40 mg absolute ethanol, heated to 50°C and filtered for sterilization, yielding a solution containing 100 mg CyA/mL as per Sandoz (Switzerland)), 4 days prior to surgery, and continued for the length of each study. Dosages were adjusted to maintain 12 hour serum trough levels around 800-1000 ng/mL, as monitored by room temperature radioimmunoassay (hereafter RIA), but below the level found to be nephrotoxic.

From experience with the earlier transplants, supplemental steroids, as used by some clinical centers (13, 25, 29, 102, 127, 165, 215, 216, 217, 230), were found to be necessary and were administered intramuscularly. A course of methylprednisolone was started at 125 mg/day for a duration of 3 days, followed by a tapering regime from 25.6 mg/day divided into 2 doses, and decreasing by 4 to 4.4 mg every 2 or more days to a maintenance dose of 4.4 mg once daily.

Perioperative Care

1. Infection control

All animals received Penlong-S^{R} [6] (0.11 mL/kg/day i.m.) beginning the day prior to surgery for a duration of 14 days and as required. This regime delivers 11,000 units each of procaine penicillin (34) and benzyl penicillin/kg/day as well as 27.5 mg of streptomycin/kg/day. This drug was chosen because it had to be administered only once daily and because of its activity against ß-hemolytic streptococcus which can be a serious pathogen in skin and soft tissue wounds. In addition, netilmicin sulfate (50 mg i.m. twice daily) was routinely instituted following infection of our first transplant with Enterobacter cloacae. It was also given for a duration of 14 days beginning the day prior to surgery and as needed. Prior to surgery, the hair on the operated limbs of the animals was clipped. Sterile surgical technique was employed during all operations and dressing changes. If there was any evidence of infection, cultures were taken and treatment with the appropriate antibiotic instituted.

2. Anesthesia and analgesia

All animals were kept fasting a minimum of 9 hours prior to surgery. Tranquillization was achieved with intramuscular ketamine hydrochloride (5.25 mg/kg) and

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[6] Rogar S.T.B., 805 Castelnau Ave., Ste-Hyacinthe, Quebec, Canada, J2S 6S4

xylazine (0.45 mg/kg) (248). Anesthesia was induced and maintained with intravenous sodium pentobarbital (16.25 to 65 mg per dose). Dosage and frequency were determined by the amount necessary to suppress reflexes and minimize muscle tone.

Levorphan tartrate was chosen as the postoperative analgesic for its effectiveness at a low dosage and its prolonged duration of action (36). It was administered at a dose of 1 mg i.m. twice daily for 5 to 7 days under official authorization.[7] To control nausea and vomitting, dimenhydrinate was administered at a dosage of 15 mg i.m. twice daily for at least 5 days post-operatively and as needed.

3. Ventilatory management

All animals where intubated with an oral cuffed endotracheal tube of appropriate size (5.0 to 6.0 mm internal diameter). Spontaneous ventilation via a Magill type circuit was allowed with inspired gas (100% oxygen) flow adjusted on the anesthetic machine[8] to provide minute ventilation (223). Adequacy of ventilation was assessed by monitoring expired carbon dioxide concentration (normal peak

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[7]Bureau of Dangerous Drugs, Health Protection Branch, Dept. of National Health & Welfare, Ottawa, Ontario, Canada, K1A 1B9

[8]Medical Section, British Oxygen Engineering, Ltd., London N.18, England. (Serial # 304952/451 and /452)

4%) with a Beckman Medical Gas Analyzer LB-2 Model 240.[9] Hand ventilation was used as required and a Bird Mark 14 positive phase ventilator[10] was available if required.

4. Hemodynamic monitoring

For the purpose of establishing a reliable intravenous line, a venous cutdown was made in either the cephalic or femoral vein. Intravenous fluids consisted of 5% dextrose in 0.45% saline administered at an average rate of 6.9 mL/kg/hour, well above the usual daily fluid intake (33), although higher rates might have been more appropriate (35). The rate was adjusted according to each animal's weight, estimated total fasting time, length of procedure, blood loss and urinary output. The latter was measured via a Foley catheter when possible (usual daily output in a young adult ranging between 150 and 400 mL) (33), otherwise the bladder was emptied using manual pressure on a regular basis to verify adequate urinary production since urethral catheterization proved to be impossible on several animals.

The heart rate was monitored by screen electrocardiogram[11] (85 (223) and 115 beats per minute (33) are considered to be hemodynamically stable rates).

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[9]Electronic Instruments Div., Beckman Instruments, Inc., 3900 River Rd., Schiller Park, Illinois, 60176, U.S.A. (Category # 149530, Serial #2015-807)

[10]Bird Corp., Palm Springs, California, U.S.A.

[11]Honeywell RM 300, Honeywell Ampletrol, Inc., 9501 Christophe Colombe, Montreal, Quebec, Canada, H2M 2E3

5. Temperature regulation

Normal rectal temperature in the baboon should be between 36.0 and 39.5°C (33, 223). Since barbiturate anesthesia can cause hypothermia, rectal temperature was monitored and maintained close to the normal range with a Gaymar Solid-State T/Pump Heat Therapy Pump, Model TP200 circulating hot water pad system[12] (or electric pad for the donor) and adequate draping.

6. Anticoagulation

Prior to sectioning of the donor flap pedicle and beginning the microvascular anastomoses, each animal received a single dose of sodium heparin (70 IU/kg i.v.). Postoperatively, acetylsalicylic acid was used for its effect on platelet aggregation as a prophylactic measure to suppress the thrombotic cycle at the level of the vascular anastomoses (42). In addition, this drug, used by some transplant centers (128) and known to reduce the inflammatory response thus assisting in controlling the effects of the immune response (197), was given for a duration of at least 6 days at a dosage of 80 mg p.o. once or twice daily.

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[12]Gaymar, 1 Bank St., Orchard Park, New York, 14127, U.S.A.

7. Dressing and splinting

Following surgery, chlorhexidine acetate gauze was applied to all incisions followed by sterile bulky dry dressings. The operated site was protected by a modified version of the rigid, custom-made, thermoplastic upper limb splint described by Rose et al (192). Dressings were changed using sterile technique and the splint cleaned at every transplant verification (usually once or twice weekly). The splint was reinforced as required and worn at all times until the first neurophysiologic studies proved that sensory recovery had occurred.

Postoperative Care

1. Cyclosporin A Radioimmunoassay

In order to monitor serum trough levels and to expedite dosage adjustments, the CyA RIA[13] was set up for use in our laboratory after consultation with an external hospital clinical laboratory[14] where the assay was in use.

Since CyA has some toxic side effects at very high serum levels, all animals receiving CyA had levels measured three times a week for the first month, twice a week for the next month, and, according to the stability of the animals and their previous results, thereafter (on the average)

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[13] Sandoz, Ltd., CH-4002, Basle, Switzerland

[14] Biochemistry Dept., Hôpital Notre-Dame, 1560 Sherbrooke St. E., Montreal, Quebec, Canada, H2L 4M1

weekly. When dosage adjustments were necessary, serum levels were monitored more frequently until they stabilized. Dosage was adjusted to maintain serum trough levels around 800-1000 ng/mL and within the 800-1500 ng/mL range found necessary for these transplants. Animals were weighed regularly.

2. Biochemistry

CyA has been found to have a number of side effects when used clinically. The most common serious side effect of high dose CyA is nephrotoxicity (8, 24, 26, 28, 29, 30, 37, 87, 102, 106, 110, 121, 127, 131, 165, 175, 177, 190, 194, 195, 204, 214, 215, 216, 217, 230, 231, 232, 233). Some authors have also reported hepatotoxicity, particularly when high dose CyA is combined with another immunosuppressant (24, 26, 28, 29, 37, 73, 87, 89, 102, 106, 110, 165, 177, 194, 204, 214, 216, 230, 231, 232, 233). These have been found to be related to high serum trough levels and reversible upon lowering or stopping the CyA therapy. Serum blood urea nitrogen and creatinine levels as well as liver function tests were therefore drawn on the same schedule as for CyA levels.

3. Hematology

Although the potential for myelotoxicity does exist (90), it seems to be less frequent than with standard immunosuppression (14, 15, 16, 37, 73, 111, 114, 127, 139, 170, 194, 230, 231) weekly complete blood cell count during the first month, and twice monthly thereafter was used to monitor the white blood cell count in view of chronic steroid therapy. Since CyA is primarily transported in plasma by the lipoprotein fraction, a lipoprotein electrophoresis was done on the first long-term transplant recipients (9, 37, 127, 140). Unfortunately, the pattern obtained was difficult to interpret in terms of its relevance to the bioavailability of the drug and so electrophoresis on the remaining animals was not undertaken.

4. Transplant technical assessment

As with all free flaps, the immediate postoperative viability of the transplant depends entirely upon extrinsic surgical factors. These include: flap design, atraumatic isolation, level of vessel repair, microanastomoses of the vessels, dressings, anticoagulants, antibiotics, and sterile technique. Problems stemming from these factors manifest themselves in failure of the flap within the first few days after surgery. The first assessment therefore was usually at 3 days postoperatively when surgical success was determined. Because the transplants were completely covered

for protection, it was impossible to verify viability on an ongoing basis. Since salvage of a transplant failing on a vascular basis was not possible, the only way to avoid failure was prevention with meticulous technique.

5. Rejection protocol

Published data suggest that CyA should, if administered at the appropriate dosage, prevent rejection as evidenced by experimental skin graft transplants (17, 50, 99, 100, 141, 142, 165, 235, 246) and limb transplants (10, 11, 92, 93, 115, 129). Rejection occurs within 2 weeks in an initially successful free flap transplant when the recipient is not immunosuppressed (198) and by 6 to 16 days (depending on the model and end point considered to be rejection) in limb transplants (i.e., transplant controls) (76, 96, 178). Thus, following initial verification, the status of the transplant was checked twice a week. When there was any concern about the flap or hand, more frequent checks were initiated.

Signs signalling possible rejection include edema, erythema, induration, mottling, vesiculation, ulceration, vascular compromise, necrosis, and eschar formation (76, 93, 96, 143, 198). With the appearance of any of these signs in some of the earlier allografts, a 2 mm punch biopsy was sent for immediate assessment by our consultant pathologist

specialized in transplantation[15] and was repeated after successful treatment of the crisis. If histologic evidence of rejection was present, with edema, cellular infiltrate, vasculitis, dermal hemorrhage, small vessel thrombosis, skin atrophy or necrosis (76, 93, 96), the plan was to institute therapy designed to save the transplant. It soon became evident that the delay between biopsy and the feedback from conventional pathology analysis was unacceptably long. In addition, it became apparent that the distinction between a primarily rejective process and a dominantly infective one was extremely difficult to make on the basis of such histological material.

With experience, clinical assessment proved to be a reliable, accurate and prompt method of determining early signs of rejection, as shown by several successful reversals of allograft deterioration. At the earliest signs of rejection, the plan was to implement systematic treatment. This consisted of increasing the CyA to a dosage at which 12 hour serum trough levels remained consistantly above 1000 ng/mL (to around 1500 ng/mL) and boosting the methylprednisolone back to the beginning of the dosage regime. A similar steroid dosage schedule is used in renal

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[15] Juergen Knaack, M.D., Pathology Dept., Royal Victoria Hospital, 687 Pine Ave. W., Montreal, Quebec, Canada, H3A 1A1, and Associate Professor of Medicine, McGill University, 845 Sherbrooke St. W., Montreal, Quebec, Canada, H3A 2T5

allograft patients and was recommended by our transplantation consultant.[16]

In addition, acetylsalicylic acid was usually restarted at a higher dose of 75-100 mg twice daily p.o. to obtain its anti-inflammatory (197) and anti-platelet activity. Some renal transplant centers utilize an anti-platelet drug both following transplantation and during rejection episodes. The rationale for this, as clarified by Kauffman, is based on the findings of several authors that platelets adhere to the vascular endothelium (128) and that both thrombosis and obliterating vascular lesions are found in rejecting allografts (1, 251). Skin allografts have also been found to undergo vascular damage during rejection with subsequent ischemia (155).

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[16]Ronald D. Guttmann, M.D., Director, Transplant Service at the Royal Victoria Hospital, 687 Pine Ave. W., Montreal, Quebec, Canada, H3A 1A1, and Professor of Medicine, McGill University, 845 Sherbrooke St. W., Montreal, Quebec, Canada, H3A 2T5

EXPERIMENTS

Stage I: Anatomy and Design of Transplant Models

Experiment #1: Anatomy of the baboon forearm and hand

The anatomy of the baboon hand resembles that of the human (6, 212, 213) with a few differences. The following description is the result of our own anatomical dissections of three upper limbs in two animals (#1,#2) (Table 1) and based on a review of the literature (116, 221).

1) General appearance The hand of a young adult Papio hamadryas anubis is significantly smaller than that of the human, measuring approximately 10-12 cm in length by 5 cm in width. The proportion of the digits to the palm is markedly different from that in the human, with a ratio of almost 1:2 in the case of the third digit in contrast to less than 1:1.5 for the same digit in humans. The thumb is short, measuring between one half and two thirds of the middle digit. As in humans, dorsal skin is loose and thin while volar skin is thick and fixed. Hairy skin is present over the entire dorsum of the hand and on the dorsum of the proximal and middle phalanges. Glabrous skin is present over the entire palm and volar aspects of all digits. The volar interdigital pads overlying the metacarpophalangeal joints are well developed as is the pulp on the finger tips.

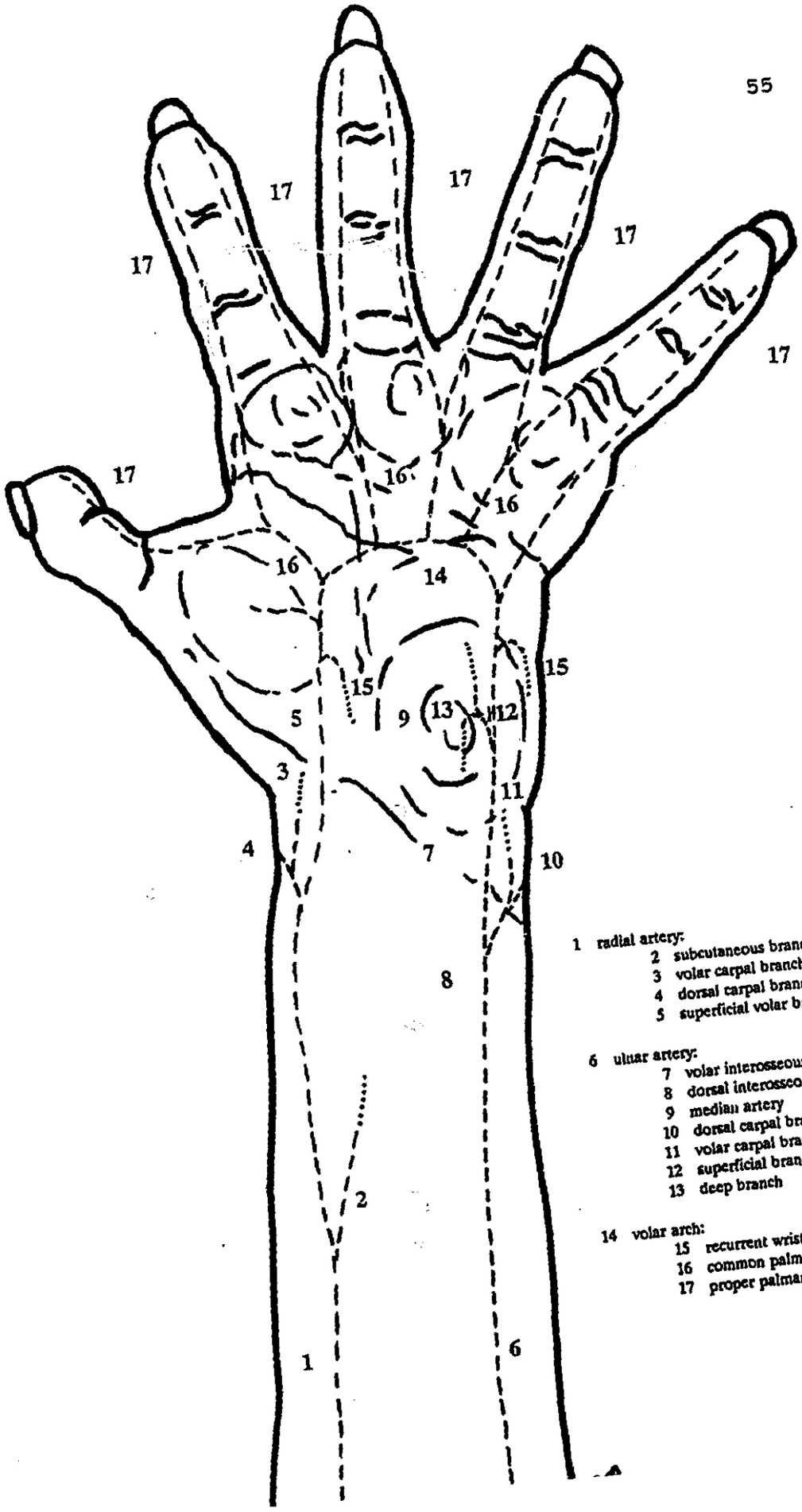
2) Vascular anatomy Arterial supply (Figure 1) to the forearm and hand is via the radial and ulnar arteries,

Table 1: Summary of animals used in experiments #1 through #6 inclusive

Animal	Date of Surgery	Type of Intervention	Transplant Survival	Animal Survival	Rejection	Problems
Baboon #1	24/08/83	Anatomic dissection R hand and forearm	-	-	-	-
	28/08/83	Design of a R PH	-	-	-	-
	30/08/83	Design of a L index NVIF L upper limb and R lower limb vasculature injected for prosection	-	-	-	-
Baboon #2	09/12/83	Design of a R index NVFF	-	-	-	-
	15/12/83	Donor L index TNVFF to #3	-	-	-	-
Rabbit	-	CyA oral administration and RIA levels	-	-	-	-
Baboon #3	15/12/83 TNVFF from #2	Recipient L index	D15 (last dressing change)	D20 (euthanasia)	Do acute	Oral CyA administration undependable

D : postoperative day
PH : (distal forearm level) pedicled hand

Figure 1: Arterial supply to the forearm and hand of Papio hamadryas anubis (left upper limb volar view).



1 radial artery:
- 2 subcutaneous branch
- 3 volar carpal branch
- 4 dorsal carpal branch
- 5 superficial volar branch

6 ulnar artery:
- 7 volar interosseous artery
- 8 dorsal interosseous artery
- 9 median artery
- 10 dorsal carpal branch
- 11 volar carpal branch
- 12 superficial branch
- 13 deep branch

14 volar arch:
- 15 recurrent wrist branch
- 16 common palmar digital artery
- 17 proper palmar digital artery

with the former being dominant in contrast to that found in most humans (172). The radial artery travels in the forearm deep to the volar border of the brachioradialis muscle near the radial nerve. It supplies the adjacent muscles and, at the junction of the middle and distal thirds of the forearm, provides a large subcutaneous branch which anastomoses distally with the volar carpal branches. At the wrist, a common stem is given off for the volar and dorsal carpal branches before the artery continues as the superficial volar branch. This artery runs deep to the abductor pollicis brevis muscle, superficial to the flexor retinaculum and flexor pollicis brevis muscle, supplying local skin and muscle. It joins the ulnar artery's superficial volar branch to form the single volar arch in the mid-palm, deep to the volar aponeurosis, at a level equivalent to the superficial volar arch in humans. No deep volar arch is present.

The ulnar artery accompanies the ulnar nerve to the wrist, between the flexor carpi ulnaris muscle superficially, and the flexor digitorum profundus muscle deep. In the forearm, in addition to vessels supplying tissues in the elbow region, the common interosseous artery is given off. This branch soon divides into volar and dorsal interosseous arteries which nourish the surrounding musculature. The volar interosseous vessel also produces the small diameter median artery which accompanies the

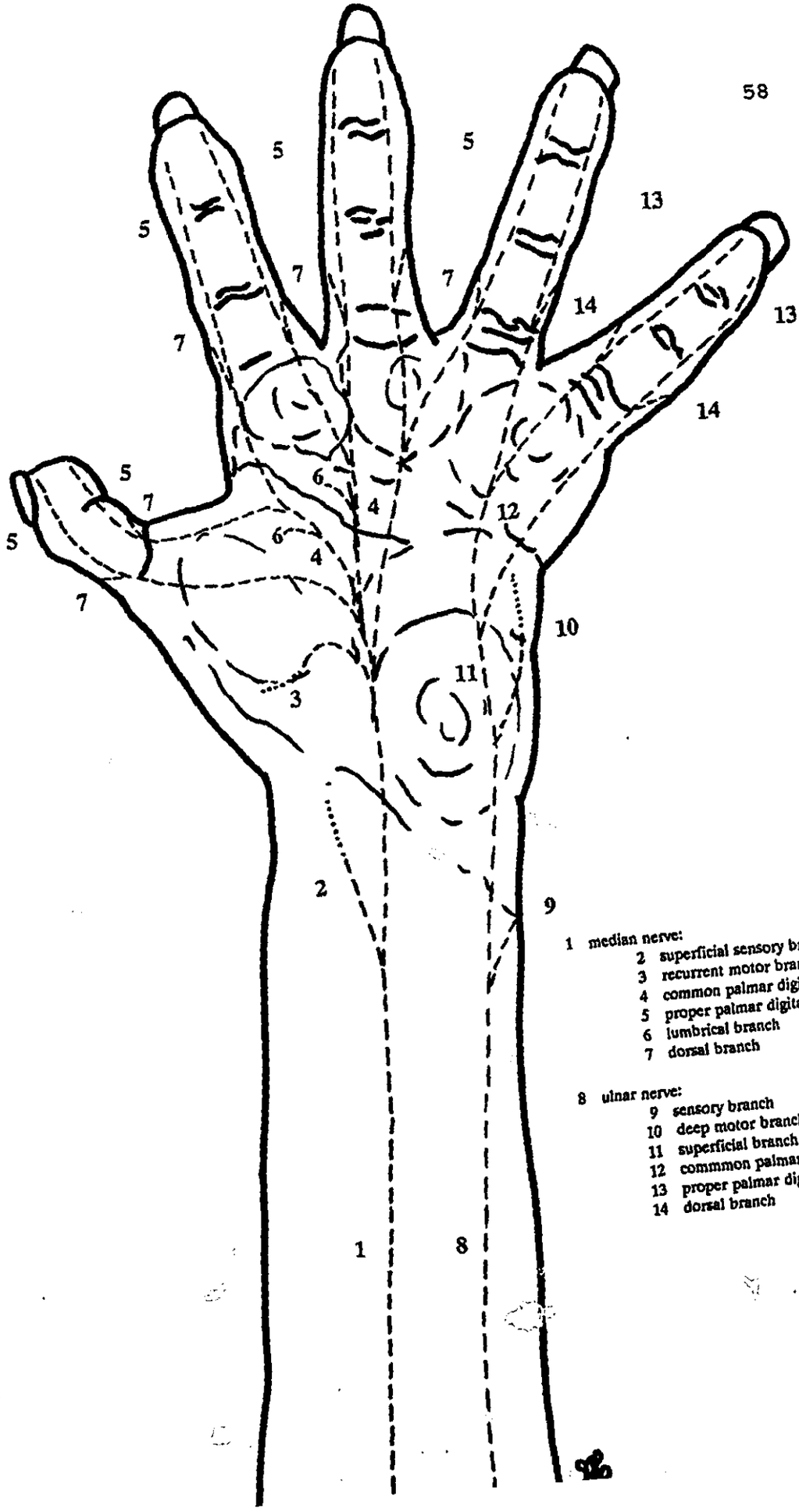
median nerve. Before crossing the wrist joint, from the ulnar artery arise the dorsal and volar carpal branches via a common origin. As it enters the hand, the ulnar artery next divides into superficial and deep branches. In its course distally, the superficial branch runs deep to the volaris brevis muscle and the volar aponeurosis to join the volar arch. The deep branch, in the meantime, ramifies on the contrahentes muscles in both proximal and distal directions.

The volar arch gives off recurrent wrist branches and common palmar digital arteries to each web space, as well as a proper palmar digital artery to the ulnar aspect of the fifth digit. In turn the arteries to the web spaces divide, as they approach the metacarpophalangeal joints, into proper palmar digital arteries, one to each side of every digit.

Venous drainage is provided by an interconnecting complex of veins originating mainly in the dorsum of the digits and coursing proximally toward the radial aspect of the wrist. There they join smaller veins draining volar structures to form a single cephalic vein (the basilic vein is absent in the baboon). In addition, small pairs of venae comitantes accompany the larger deep arteries.

3) Neural supply The nerve supply to the forearm and hand is similar to that in humans (Figures 2 & 3) with a few exceptions. While running between the flexor digitorum superficialis and profundus muscles in the forearm, the

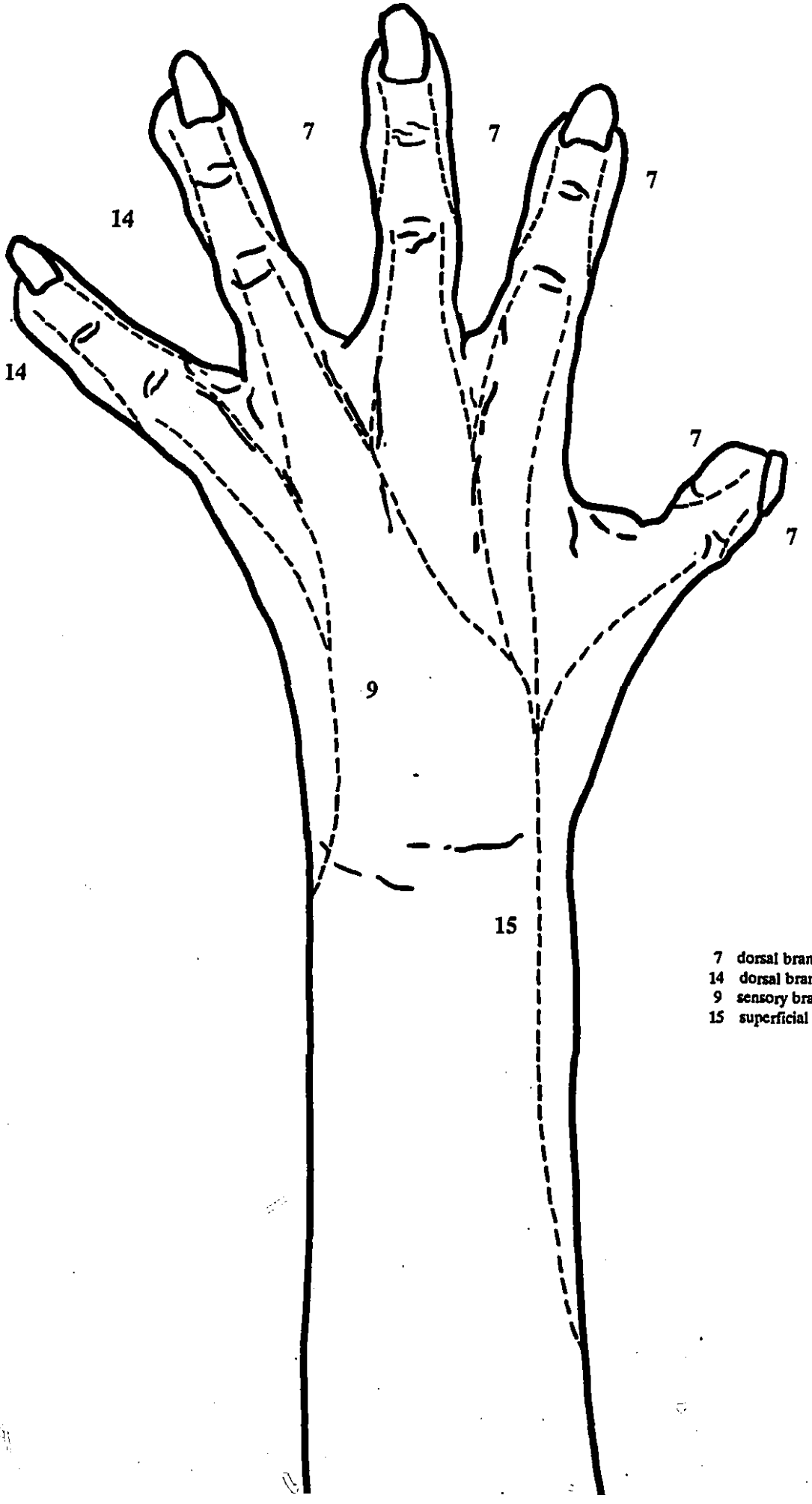
Figure 2: Nerve supply to the forearm and hand of Papio hamadryas anubis (left upper limb volar view).



1 median nerve:
- 2 superficial sensory branch
- 3 recurrent motor branch
- 4 common palmar digital nerve
- 5 proper palmar digital nerve
- 6 lumbrical branch
- 7 dorsal branch

8 ulnar nerve:
- 9 sensory branch
- 10 deep motor branch
- 11 superficial branch
- 12 commmon palmar digital nerve
- 13 proper palmar digital nerve
- 14 dorsal branch

Figure 3: Nerve supply to the forearm and hand of Papio hamadryas anubis (left upper limb dorsal view).



7 dorsal branch of the median nerve
14 dorsal branch of the ulnar nerve
9 sensory branch of the ulnar nerve
15 superficial branch of the radial nerve

median nerve provides motor innervation to the pronator teres muscle and all of the long flexors except for the flexor carpi ulnaris muscle. As the nerve approaches the wrist, the superficial sensory volar branch is given off and the median nerve passes through the carpal tunnel deep to the volaris longus muscle in a position slightly radial and superficial to the digital flexor tendons. Upon exiting the carpal tunnel it provides the recurrent motor branch to the thenar muscles (except to the deep head of the flexor pollicis brevis and adductor pollicis muscles, as in humans), and the median nerve divides into three common palmar digital nerves to the first three web spaces, as well as a radial proper palmar digital nerve to the thumb. The nerves to the first two web spaces also supply a motor branch to each of the radial two lumbrical muscles. The former in turn divide into proper palmar digital nerves in the distal third of the palm supplying sensation to adjacent sides and corresponding distal dorsum of the first four digits as does the radial proper palmar digital nerve to the thumb.

The ulnar nerve travels in the forearm between the flexor carpi ulnaris and flexor digitorum profundus muscles accompanied for most of its more distal course by the ulnar artery. After a sensory branch passes dorsally, the nerve travels with the artery into the proximal palm where a deep motor branch innervates the hypothenar, ulnar two lumbrical,

interossei muscles, as well as the contrahentes manus group, adductor pollicis, and deep head of the flexor pollicis brevis muscles. The superficial branch divides into an ulnar proper palmar digital branch and a common palmar digital nerve in the middle third of the palm. The latter divides in the distal palm into two proper palmar digital nerves supplying sensation to adjacent sides and corresponding distal dorsum of the fourth and fifth digits as does the ulnar proper palmar digital nerve to the fifth digit. The sensory branch of the ulnar nerve supplies the ulnar side of the dorsum of the hand and part of the dorsum of the ulnar one and a half digits.

The deep branch of the radial nerve, after piercing the supinator muscle which it supplies, ramifies to innervate the extensor compartment of the forearm. The superficial branch of the radial nerve accompanies the radial artery deep to the volar border of the brachioradialis muscle. Upon approaching the wrist it takes a radio-dorsal direction subcutaneously to supply the radial aspect of the dorsal hand and portions of the dorsum of the first through radial half of the fourth digits.

4) Musculoskeletal system Although there are some differences in morphology, shape, proportion and size (the ulna and radius in the distal forearm, 3-4 cm proximal to the wrist measure approximately 0.7 and 1.0 cm in diameter, respectively), all bones present in the baboon forearm and

hand are equivalent to those in humans with only two exceptions. First, there is an additional carpal bone in the baboon named os centrale which fuses to the scaphoid before birth in humans and is therefore indistinguishable. This bone in the baboon articulates with the scaphoid in the proximal carpal row and the capitate, trapezium, and trapezoid in the distal row. The second difference is the number of sesamoid bones. In the baboon there are a total of nine (two at each metacarpophalangeal joint of digits two through five and only one in the thumb) whereas in humans there are usually no more than five (one at the interphalangealjoint of the thumb and the metacarpophalangeal joints of digits two and five, and two at the metacarpophalangeal joint of the thumb).

Some differences in the neuromuscular system of the forearm and hand are also present. The only neural modification is in motor innervation of the flexor digitorum profundus muscle which is entirely median innervated in the baboon as opposed to both median and ulnar innervated in humans. Although some muscular origins and insertions differ slightly, the most significant changes in the baboon are the missing and additional muscles present. The only two muscles seen in humans but absent in the baboon are the flexor pollicis longus muscle which is replaced by a slip of flexor digitorum profundus muscle to the thumb, and the extensor pollicis brevis muscle. There are a number of

muscles in the baboon not identifiable in humans. The extensor digiti medius muscle arises from the distal aspect of the dorsal ulna in common with the extensor digiti indicis muscle (also known as the extensor indicis proprius muscle), terminates on the dorsum of the third proximal phalanx, and is innervated by the radial nerve. The extensor digiti anularis muscle, on the other hand, originates on the lateral epicondyle of the humerus in common with the extensor digiti minimi muscle, inserts on the ulnar side of the dorsal proximal phalanx of the fourth digit and is also innervated by the radial nerve. In the hand proper, three contrahentes manus muscles originate from the contrahentes tendon which extends from the bases of the second and third metacarpals to the head of the third metacarpal. The first inserts on the ulnar side of the second proximal phalanx, the second on the radial side of the fourth proximal phalanx, and the third on the radial side of the fifth proximal phalanx. All three derive their innervation from the deep branch of the ulnar nerve. Although humans possess seven interossei muscles, the baboon can have anywhere from seven to 11. Because of their location, there is some disagreement in nomenclature, depending on the author, as to their distribution between the dorsal and volar surfaces of the hand. Some claim that there are four dorsal and anywhere from three to seven volar interossei (221) while others believe these numbers to be

reversed (116). The remaining characteristics of the musculoskeletal system are similar in the two species.

5) Summary After undertaking dissections of the baboon forearm and hand while extensively reviewing the anatomy in the literature, the similarities and differences between the baboon and human were clarified. It was possible to formulate a plan and design a neurovascular free flap and hand transplant model while becoming familiar with the particularities of the baboon distal upper limb.

Experiment #2: Design of a neurovascular island flap

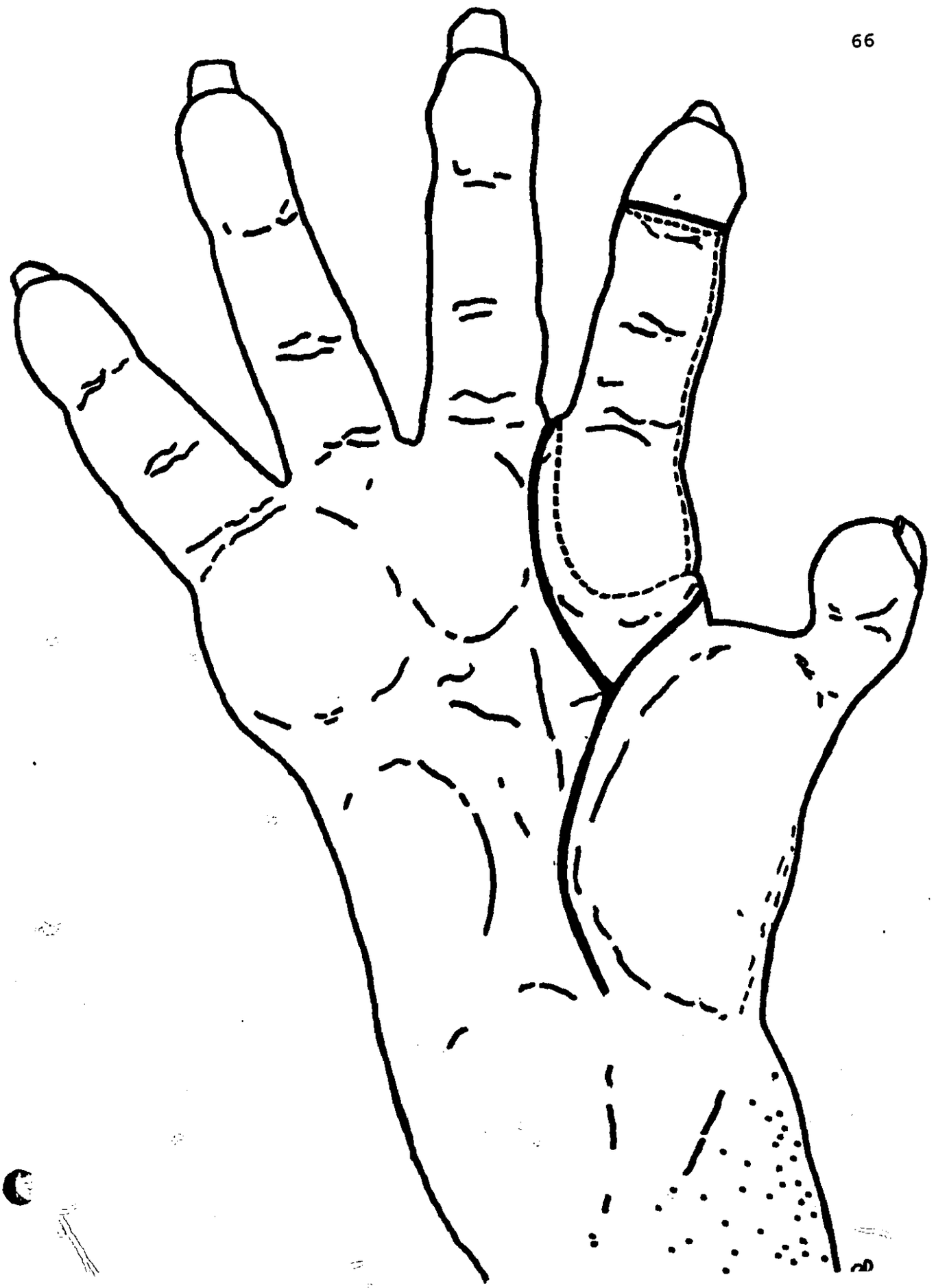
1) Background The purpose of this experiment was to design a neurovascular island flap (hereafter NVIF) in the baboon. A modification of the digital island flap described by Littler (145) was chosen in view of the following characteristics: 1) glabrous and hairy skin, 2) pure sensory innervation (proper palmar digital nerves), 3) consistent vascular supply (proper palmar digital artery / dorsal vein), and 4) adequate pedicle length and anastomotic size at the mid-metacarpal level.

The digital island flap was originally developed for the reconstruction of digits in which soft tissue or nerve damage limited adequate sensory function. By transferring skin and subcutaneous tissue on a neurovascular pedicle, it became possible to restore sensation to a vital region (pinch surface of the thumb or index) at the expense of that

of a less important area (ulnar aspect of the long or ring finger). The modifications made for the purposes of this project included degloving (removing the entire skin / soft tissue coverage with its neurovascular supply and leaving behind the osteo-tendinous structures) of the entire digit with incorporation of the radial most interdigital pad and the skin overlying the dorsum of the metacarpophalangeal joint, plus the preservation of a dorsal digital vein to insure adequate venous drainage.

2) Design After thorough review of both human and baboon hand anatomy and dissection in the baboon, a NVIF was designed on the right second digit of a single animal (#1) (Table 1) using the following technique: Under general anesthesia, the baboon was placed in the supine position with the upper limb abducted. The proposed skin incisions were made to encompass the entire soft tissue coverage of the second digit from proximal to the metacarpophalangeal joint in a distal direction, excluding the nail structures (Figure 4). A curvilinear line was traced along the thenar crease in the palm to join the proximal point of the V-shaped incision planned in the skin crease surrounding the radial interdigital pad. Another V-shaped marking was made on the dorsum of the hand, enclosing the skin over the second metacarpophalangeal joint and connecting with the ends of the volar V in the first and second web spaces. To complete the design, a line was drawn along the radial mid-

Figure 4: Design for the NVIF (animal #1).



lateral axis of the second digit to the distal interphalangeal joint where a circumferential incision demarcated the distal end of the flap.

The incisions were planned to facilitate access to the common palmar digital artery supplying the second web space, both proper palmar digital nerves to the index in the palm, and the dorsal vein draining the flap as it courses proximally towards the cephalic vein. The flap itself was believed to represent an axial pattern blood supply as suggested by its anatomy, with the possibility of incorporating its sensory nerve supply.

The dissection was begun in the palm under tourniquet control. The proper palmar digital artery to the ulnar side of the second digit was isolated, after ligating and dividing the branch to the radial side of the third digit, to its origin from the radial component of the single volar arch, via the common palmar digital artery to the second web space. This digital artery was chosen over the one supplying the radial side of the digit for two reasons: the artery supplying the radial side proved to be consistently smaller during all dissections, and the longitudinal incision required to raise the flap from the underlying tendino-skeletal framework, while avoiding damage to the neurovascular supply, would have been more difficult to handle in the confines of the second web space. Arterial size approaches a diameter of 0.8 mm at the mid-metacarpal

level. The proper palmar digital nerves were separated from adjacent fascicles by neurolysis under magnification to the level of the midpalm. A large dorsal digital vein was isolated from the edge of the dorsal flap in a proximal direction where its diameter approaches 1 mm.

The incisions were completed and the flap raised from distal to proximal at the level of the flexor sheath and extensor tendons based exclusively on its neurovascular pedicles. The tourniquet was released and hemostasis secured with the bipolar cautery and hemoclips. After acute evaluation of the circulation, the flap was returned to its bed and sutured using 4-0 polyglycolic acid simple interrupted and simple continuous sutures.

3) Results This procedure confirmed adequate short term blood supply in the NVIF. When the flap was raised, based exclusively on its neurovascular pedicles, the presence of normal color, amount, and briskness of bleeding from its deep surface and a stab wound to its distal epidermal surface proved the presence of satisfactory circulation.

4) Summary This experiment was the first step toward the design of an NVFF. It helped to clarify the anatomy, including the distribution of the neurovascular supply to the soft tissues of the second digit, and provide evidence that a viable neurovascular flap design was achieved. As this flap evolved through the subsequent phases of the

study, minor improvements were made in the design, but the essential pattern stayed the same.

Experiment #3: Conversion of the neurovascular island flap to a neurovascular free flap

1) Design In this experiment, the NVIF previously described was converted to a NVFF and replanted in-situ to evaluate its viability. The incisions were planned as described in Experiment #2 with two modifications. First, to avoid creating a large area of skin and soft tissue with questionable blood supply at the fingertip, the distal-most incision was altered. Instead of taking a course circumferentially at the level of the distal interphalangeal joint, the incision was moved to the circumference of the nail, and 3 mm proximal to the eponychium. It was felt that the ungual tissues themselves would survive due to their close proximity to the dorsal periosteum of the distal phalanx as found in humans (255). The second change was the addition of a longitudinal incision on the dorsum of the hand from near the tip of the V-shaped flap, proximally. This allowed easier access to the draining vein, permitting the anastomosis of a more proximal vessel of larger caliber (approximately 1.2mm versus 1.0mm and less more distally).

The following technique was therefore used on the right second digit in a single animal (#2) (Table 1): Under general anesthesia, the baboon was placed in the supine

position with the upper limb abducted. The proposed skin incisions were delineated as in Experiment #2 including the two alterations described above. Dissection was begun in the palm under tourniquet control (Figure 5). The proper palmar digital artery to the ulnar side of the second digit was isolated to its origin from the radial component of the volar arch in the proximal third of the palm, where its diameter is around 1 mm, sectioning and ligating all necessary branches (the proper palmar digital artery to the radial side of the third digit, the ulnar side of the volar arch and the common palmar digital artery to the first web space). The proper palmar digital nerves were identified, isolated, labelled, and sectioned in the midpalm. The dorsal vein was located and dissected as far proximally as possible through the longitudinal incision on the dorsum of the hand. The flap was raised superficially to the extensor apparatus and flexor tendon sheath from distal to proximal while protecting the neurovascular structures. Heparin was administered intravenously. The artery was clamped with an Acland 2V Vessel Approximator[17] in the proximal palm and sectioned distally while the vein was similarly transected at the wrist. The flap was completely removed from its bed for approximately 15 minutes (Figure 6) while the tourniquet was let down and hemostasis undertaken. The flap was then returned to the donor site and anchored with a few sutures.

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[17] S & T Chirurgische Nadeln, 7893 Jestetten, Federal Republic of Germany

Figure 5: Dissection in the palm at surgery in preparation for the NVFF (animal #2). The second digit is to the L, the thumb to the top, and the wrist to the R. Two pieces of pale background material are placed beneath the proper palmar digital nerves to the second digit (L). A piece of pale background material is placed beneath the radial component of the volar arch (center R).

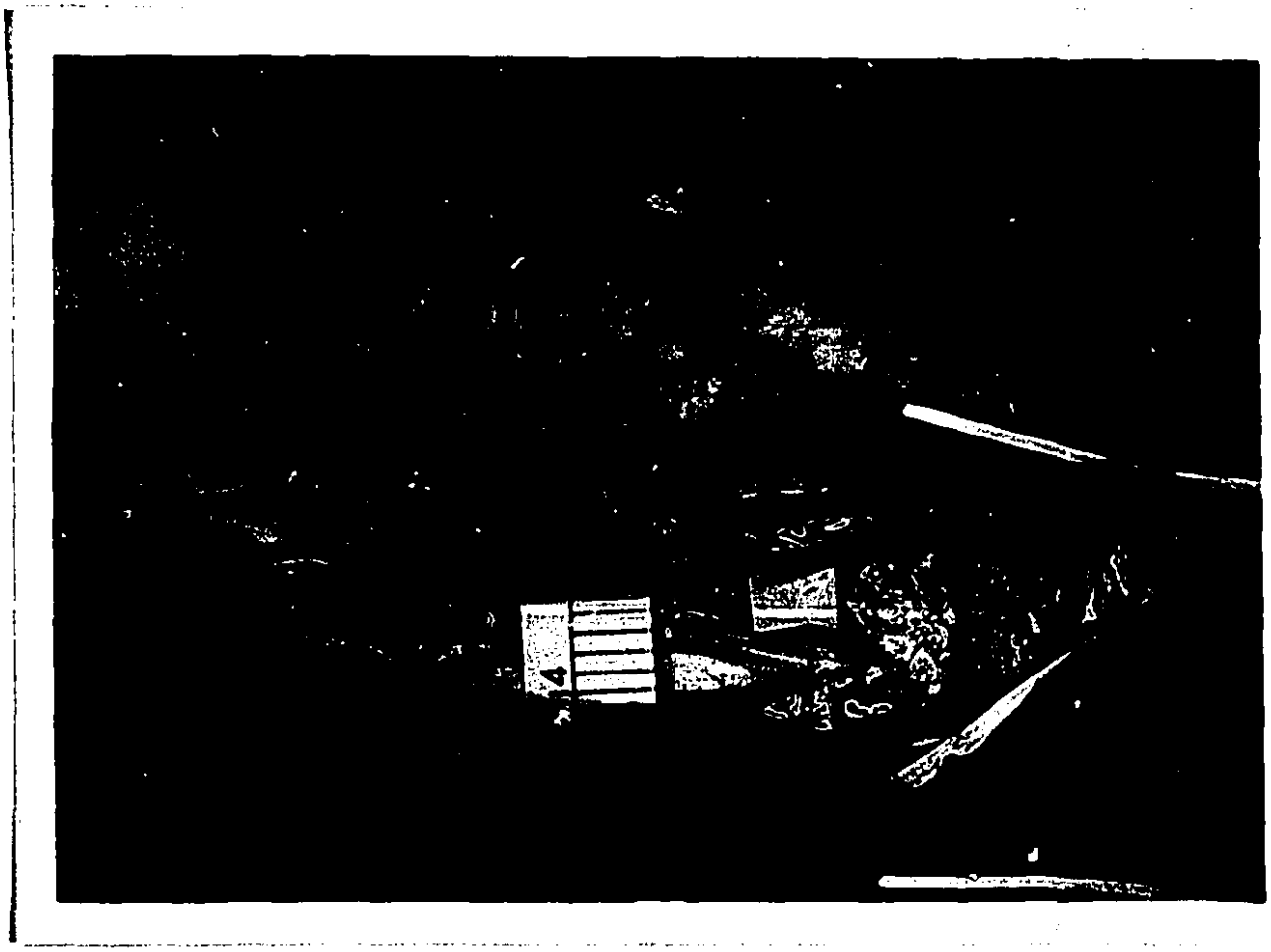
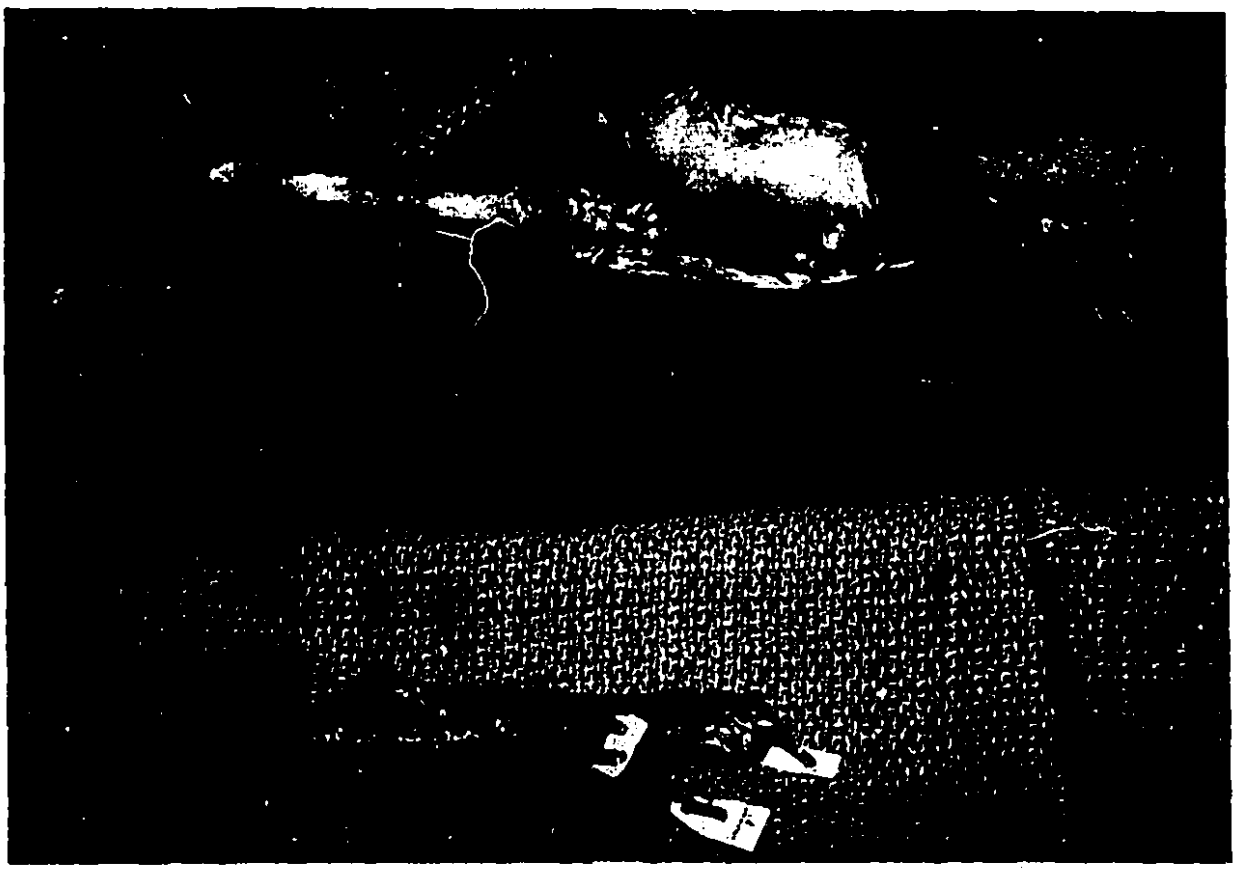


Figure 6: NVFF removed from its bed at surgery (animal #2). A piece of pale background material is placed beneath the two proper palmar ditital nerves and another beneath the dorsal vein. A portion of calibrated ruler is placed beneath the radial component of the volar arch (proximal end of the arterial supply to the NVFF).



The vascular anastomoses were undertaken with a Zeiss OpMi 1 operating microscope[18] and standard microsurgical technique (62). Nine to 12 simple interrupted stitches of 10-0 nylon on a 75 μm needle were necessary. One percent lidocaine without epinephrine was used to irrigate each microvascular anastomosis in order to prevent early vasospasm after release of each set of clamps (62). Once the flap was revascularized, the appropriate nerve bundles were aligned and the ends cut back as necessary. Three to five 10-0 nylon simple interrupted epineurial stitches on a 75 μm needle completed the repair (65). Hemostasis was secured with the bipolar cautery and hemoclips prior to suturing the flap into its bed with 4-0 polyglycolic acid simple interrupted and simple continuous stitches. A dressing and thermoplastic splint were applied.

2) Intraoperative results Intraoperatively, the success of the microanastomoses was shown by the patency test (62). In addition, normal color, briskness, and quantity of bleeding were present from the undersurface of the flap before hemostasis and from a stab wound to the distal epidermis.

3) Postoperative results Postoperatively, long-term viability of the flap was confirmed at 3 and 6 days by evidence of normal color, skin integrity, and temperature, with only a mild to moderate degree of edema. This was due

[18]Carl Zeiss Canada, 45 Valleybrook Dr., Don Mills, Ontario, Canada, M3B 2S6

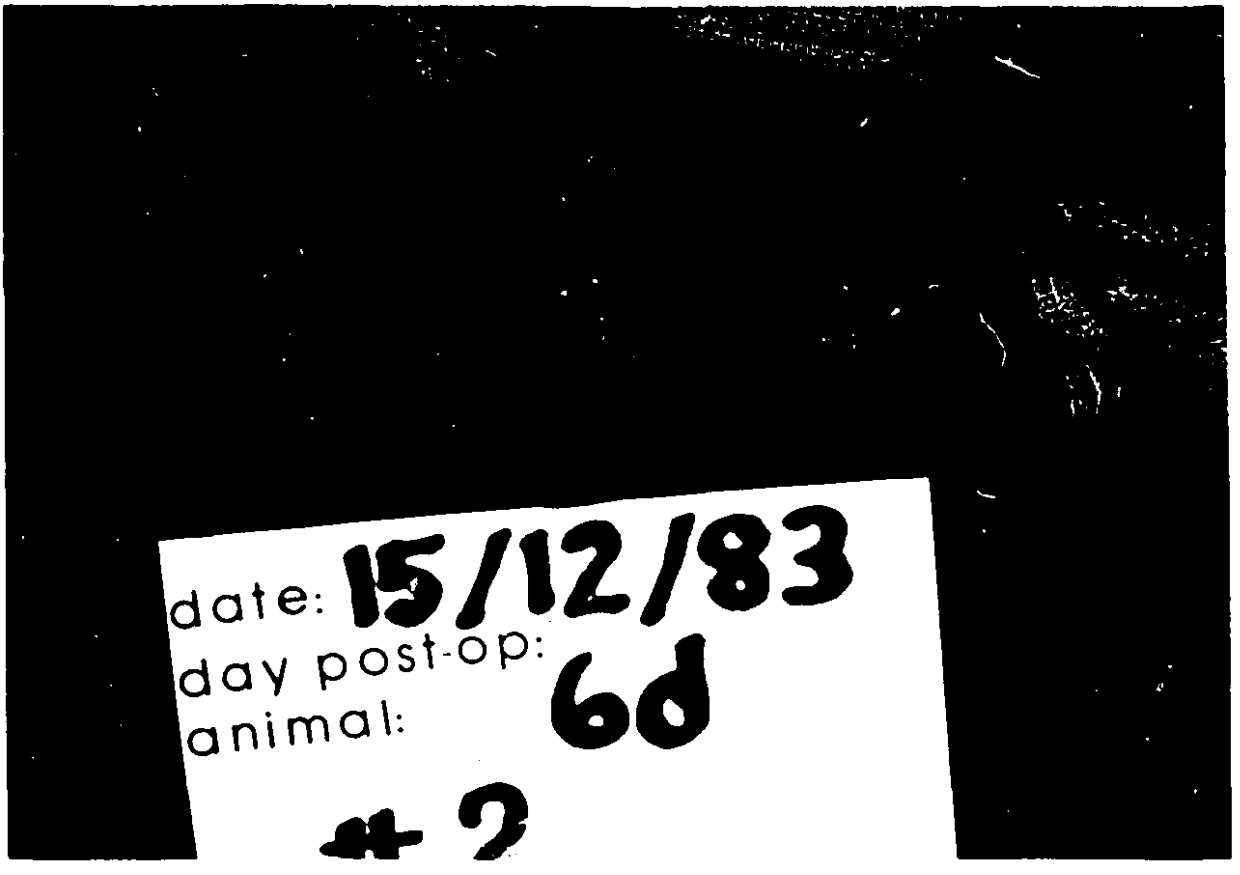
in part to the constant dependency of the flap. Bleeding from a distal stab wound to the volar surface was consistently normal in briskness, amount, and color (Figure 7).

4) Summary Since vascular failure usually occurs within the first few postoperative days, survival of this flap at 6 days indicated technical success and the achievement of a viable neurovascular free flap design. Although this model would be further refined during the transplantation experiment, it incorporated all the elements necessary for those wishing to study sensory reinnervation of transplanted tissue.

Experiment #4: Design of a distal forearm level neurovascular pedicled hand model

1) Background The purpose of this study was to design a pedicled version (without transection of arterial supply, venous drainage or neural distribution) of a hand transplant model in the baboon. A modification of the commonly used hand replant technique (63, 86, 238) was used for the following features: 1) presence of both skin and muscle, 2) sensory and motor nerve supply, 3) consistent vascular supply (radial artery/cephalic vein), 4) adequate pedicle length and anastomotic size at the distal forearm level, and 5) a significant antigenic load comprised of multiple tissues with varied antigenicity.

Figure 7: NVFF bleeding from a distal stab wound 6 days post-operatively (animal #2).


date: 15/12/83
day post-op: 6d
animal: # 2

The modifications instituted in the baboon for the purpose of this project included basing the hand on a single artery and vein. Although two arteries were available (radial and ulnar), it was hoped that using only the dominant radial would provide sufficient circulation via the single arterial arch. In addition, it was hoped that the cephalic vein alone would provide sufficient drainage. If successful, this vascular system would be advantageous in: 1) decreasing operative time (an important factor since the NVFF replant took around 15 hours including surgery and immediate perioperative care, with the expectation that a hand transplant (hereafter HT), with only one available surgical team, would be expected to last 17-24 hours), and 2) avoiding engorgement of the hand since only a single large caliber vein was present (cephalic), the venae comitantes being of small caliber (less than 1 mm at the distal forearm).

2) Design After thorough review of both human and baboon hand anatomy and dissection in the baboon, acute viability of a right hand, based on a neurovascular pedicle at the distal forearm level was undertaken in a single animal (#1) (Table 1). The following technique was used: Under general anesthesia, the baboon was placed in the supine position with the upper limb abducted. A circumferential skin mark was made on the forearm approximately 4 cm proximal to the styloid process.

Additional longitudinal incisions on the volar and radial sides of the forearm were made as required during the surgery to provide additional exposure. Dissection was begun on the volar surface under tourniquet control. Skin flaps were raised in proximal and distal directions sectioning and coagulating all small vessels encountered. The radial and ulnar arteries as well as the median and ulnar nerves were isolated and dissected over a distance of approximately 2 cm. The ulnar artery and the venae comitantes of both the ulnar and radial arteries were ligated using 4-0 polyglycolic acid and 6-0 nylon sutures respectively and hemoclips, then sectioned. The radial artery, measuring approximately 1.2 mm in diameter was preserved along with the ulnar and median nerves. The flexor tendons were individually identified and sectioned 4 cm proximal to the wrist joint. The hand was turned over and dorsal dissection undertaken. The skin flaps were raised and all small vessels sacrificed except for the cephalic vein, measuring between 1.5 and 2.5 mm in diameter, which was freed from surrounding structures over a 2 cm distance. The superficial branch of the radial nerve was isolated, dissected, and preserved. The extensor tendons were individually identified and transected 4 cm proximal to the wrist. The interosseous membrane, proximal portion of the pronator quadratus muscle, posterior and anterior interosseous arteries and nerves were all sectioned 4 cm

from the wrist. The periosteum of the radius and ulna was incised at the same level and elevated from the underlying bone for a total distance of 1 cm. The forearm bones were then osteotomized with an electric saw excising 0.5 cm.

Fixation of the radius and ulna was undertaken using small 6-hole Synthes dynamic compression plates, and 2.7 mm screws.[19] The tendons were each repaired using a modified Kessler-Mason-Allen stitch for those with a larger surface area (211) and several figure of eight 4-0 polyester stitches for each of the rest. The skin was closed using 4-0 polyglycolic acid simple interrupted and simple continuous stitches. A dressing and cast were applied.

3) Intraoperative Results After sectioning all but the neurovascular structures, good blood circulation was confirmed in the pedicled hand. Bleeding from a stab wound to the volar surface of the second digit was normal in color, amount, and briskness and remained so when reevaluated at the end of the procedure.

4) Postoperative results Postoperative viability of the hand was confirmed 2 days later by evidence of normal color, skin integrity, and temperature with only a mild to moderate degree of edema. This was felt in part to be secondary to constant dependency of the hand. Within 48 hours, once the animal had awoken fully from the anesthetic,

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[19] Synthes (Canada), Ltd., 6790A Pacific Circle, Mississauga, Ontario, Canada, L5T 1N8 (Catalogue #244.06 and Catalogue #202 series)

she had gnawed through the cast and partly reopened the forearm incision.

5) Summary Although some modifications in the operative procedure would still be necessary to successfully transplant a hand, a viable model for this purpose was designed. Acute circulation proved to be adequate based on a single artery and sole vein. Incorporation of an adequate nerve supply was possible by including the median, ulnar and superficial branch of the radial nerves. Solid internal fixation of the forearm bones was achieved using dynamic compression plates and screws.

It became evident from this experiment that conventional casting would not guarantee sufficient protection to the operated limb. Reviewing the literature provided alternatives for the subsequent studies (74, 192).

Stage II: Immunosuppression

After a review of the literature and discussions with Dr. Ronald D. Guttmann, it became clear that transplantation of limbs or parts thereof in a higher species would require powerful immunosuppression. Cyclosporin A was the drug of choice for use in investigating the transplantation of tissues previously rejected using other drugs or methods. Because the new medication was in high demand, but still experimental, the only source of supply was through sponsorship by Sandoz (Switzerland). After application,

approval of the project was granted and a supply of pure CyA powder provided throughout the length of the study by Sandoz (Switzerland) and its local branch, Sandoz (Canada).[20]

In order to use the CyA effectively, levels had to be monitored for several key reasons: 1) to help maintain therapeutic levels, 2) to determine the therapeutic range necessary for successful transplantation of the two models designed, 3) to attempt to define the lower end of the toxic range in the baboon (209), 4) to allow eventual study of the relationship between dosage, method of preparation, method of administration, and levels in the baboon (209), and 5) to assist in delineating parameters for the rejection protocol. In order to minimize time delays and because of limited access to CyA level testing, it was decided to set up the assay in our research laboratory. The RIA method of analysis for CyA was the simplest and most widely used, and therefore an RIA kit for CyA determination was purchased from Sandoz (Canada). It was set up for trial use according to instructions provided in the kit.

Experiment #5: Cyclosporin A in the rabbit

The purpose of this experiment was to provide a means for trying out the CyA RIA kit. The rabbit was chosen for several reasons: 1) gavage feeding permitting oral dosing of the drug as recommended by Sandoz (Canada), 2) relatively

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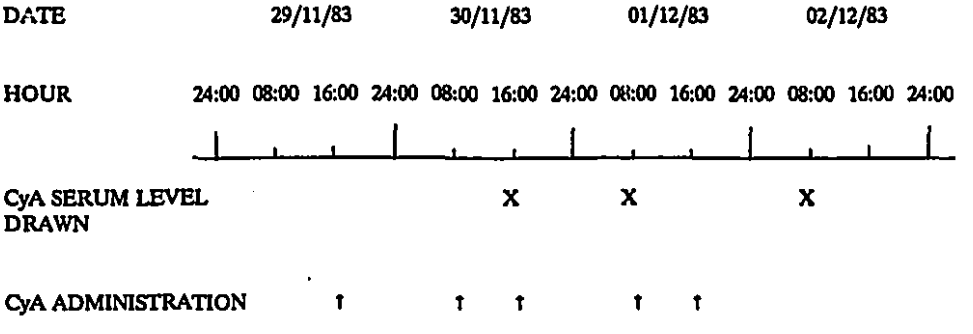
[20]Sandoz Canada, Inc., 385 Bouchard Blvd., Dorval, Quebec, Canada, H9S 1A9

low body weight avoiding the necessity for large amounts of the drug, and 3) adequate animal size for easy repeated blood sampling without danger to the animal.

1) Administration The CyA for this experiment was prepared according to the recommendations of Sandoz (Switzerland) in olive oil at a concentration of 25 mg/mL stirred for 2 hours while heated to 80°C. Cyclosporin A was administered orally to a single rabbit (Table 1) using gavage feeding at a dosage of 18/mg/kg/day (well within the dosage range of 5-50 mg/kg/day found effective in most laboratory animals (2, 232) divided twice a day for a total of five times (Figure 8).

2) Radioimmunoassay The assay was set up according to the kit instructions from Sandoz (Switzerland). Levels were drawn prior to the third and fourth doses, and 12 hours after the fifth dose of CyA. The results are indicated in Table 2. In the case of sample #1, testing was done in duplicate in order to determine whether the results varied according to the type (species) of serum used for the procedure. In one run of the assay, the tracer for the rabbit serum was made with rabbit serum and the result read off a standard curve prepared with rabbit serum, whereas in the other run both the tracer and standard curve for the same rabbit sample were prepared with human serum. Thus, two results could be obtained for this sample and it would

Figure 8: Timing of CyA administration and serum levels in the rabbit.


DATE
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CyA SERUM LEVEL DRAWN
X
X
X
CyA ADMINISTRATION

Table 2: Cyclosporin A serum levels in the rabbit

Specimen #	Result
1	< 25 ng/mL with full rabbit procedure 50 ng/mL with full human procedure
2	≤ 25 ng/mL with full rabbit procedure
3	≤ 25 ng/ml with full rabbit procedure

be possible to assess the influence of the type of serum used on the result of the assay.

3) Summary The results although consistently low were measurable and permitted the first uses of the assay kit. In addition, they alerted us to the finding that a single sample did not give identical results with the full rabbit procedure and the full human procedure. Therefore, in Experiment #6, any samples sent out to a clinical laboratory for external verification would have to be run internally with both procedures. This would allow equivalent results to be compared in order to determine the accuracy of our assaying technique. After discussion with several specialists of the RIA technique, it was decided that the CyA serum level result to be used for the purpose of monitoring levels and adjusting dosage should be the one done with the full species specific procedure. In other words, the results from assays done on baboons would be with baboon tracer on a baboon derived standard curve. This decision was supported by the fact that human samples are always run with a full human procedure and the finding that running the same sample with another species' procedure does not provide the same result.

Experiment #6: Cyclosporin A in the baboon

In this experiment a single newly designed NVFF was transplanted in order to perfect all aspects pertaining to

the surgery, care, and immunosuppression of the baboon. Some of the elements requiring evaluation included: the mode of administration of the CyA, the dosages of CyA required to achieve adequate serum levels, details of running the RIA, perioperative animal care, operative technique, splinting, postoperative animal care, plus infection and rejection protocols.

This experiment was therefore divided into two parts. In the first part, oral and intramuscular administration of CyA in the baboon was attempted with adjustments of serum levels. In the second part, a transplanted neurovascular free flap (hereafter TNVFF) was undertaken.

1) Part I: Cyclosporin A

A) Administration Since oral administration of CyA was recommended by Sandoz (Canada), a single baboon (#3) (Table 1) was used to develop techniques for this purpose. Cyclosporin A was prepared as in Experiment #5. In order to administer 9 mg/kg twice a day, the same dosage as given to the rabbit in Experiment #5, a large volume of the mixture was required (4.1 mL at each intake for an animal weighing 11.3 kg). The large volume as well as the mixture's insolubility in aqueous media created problems in feeding the CyA to the baboon. Various foodstuffs were tried but proved unsuccessful, primarily on the basis of volume and the inability to insure intake of the full dose.

Intramuscular administration was therefore initiated. Cyclosporin A was prepared according to the recommendations of Sandoz (Switzerland) for intramuscular injections in animals. In a mixture of 40 mg absolute ethanol and 810 mg Miglyol 812 heated to 50°C, 100 mg of CyA were dissolved. This mixture was then filtered for sterilization and yielded a product containing 100 mg CyA/mL. This was administered at a dosage of approximately 9 mg/kg twice a day intramuscularly.

Upon discovering that Miglyol 812 was edible and completely absorbed from the gastrointestinal tract (80), Sandoz (Canada) approved trying to administer the intramuscular preparation orally, requiring much smaller volumes (since it was 4 times as concentrated as the olive oil formula). This was begun on day 21, but, despite the smaller volumes, the eating habits of the baboon did not provide dependable intake and we resolved to reinstitute intramuscular administration in the remaining experiments.

B) Radioimmunoassay Serum trough levels were measured using the full baboon procedure on the average of three times per week and dosage adjustments were made to try to keep the levels within the therapeutic range, recommended by Sandoz (Canada), and felt to be appropriate at the time, of 50 to 200 ng/mL (127, 233) (Table 3). Some samples were run with the full human procedure as well in order to permit a

Table 3: Trough levels of CyA on various dosage regimes in baboon #3

Day	Dose*		Serum Trough Level		
	Full Baboon Procedure		Full Human Procedure		
			Our Laboratory	External Laboratory[1]	
	(mg/kg)	(ng/mL)	(ng/mL)		(ng/mL)
3	8.9 i.m.**	150	30		28
	repeat	180		repeat	40
7	9.7 i.m.	200	190		354
	repeat	220			
9	9.4 i.m.	170			
	repeat	205	210		190
11	8.9 i.m.	100			
	repeat	135			
14	9.1 i.m.	250	190		495
				repeat	470
16	9.4 i.m.	240	280		290
	repeat	255			
18	9.7 i.m.	390***			
21	11.4 p.o.	130			
23	14.3 p.o.	270			

* : prepared in Miglyol 812 and reflecting the dosage given 12 hr previous to the trough sample taken and the duration of treatment.

** : prior to this sample, the animal had received 2 i.m. doses of CyA prepared in Miglyol 812 and portions of 2 p.o. administrations of CyA prepared in olive oil.

*** : some technical difficulties made this result questionable.

[1]Biochemistry Dept., Hôpital Notre-Dame, 1560 Sherbrooke St. E., Montreal, Quebec, Canada, H2L 4M1

comparison of our methods of assay to an external control[21] (Table 3).

All samples sent to the external clinical laboratory[21] were identified only by number. Two samples were sent in duplicate to assess their degree of variability. If the samples from day 7 and day 14 are excluded, there was less than a 10% difference between the results from the two laboratories. The external laboratory had a similar degree of variation between its results for a single sample. It was unclear why the samples from day 7 and day 14 yielded such a discrepancy in results between the two laboratories, but it was evident that some further experience with the assay was still required before consistency could be expected. Nevertheless, it was possible to maintain values within the intended therapeutic range, although attempts at boosting the serum trough level to 400 ng/mL with oral dosing proved difficult.

C) Summary This part of the experiment helped provide some experience with CyA, as well as its administration and RIA. A new method for the oral administration of the drug was tried, but did not prove to be dependable.

Intramuscular administration was shown to be useful, and although not ideal, did provide accurate administration.

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[21] Biochemistry Dept., Hôpital Notre-Dame, 1560 Sherbrooke St. E., Montreal, Quebec, Canada, H2L 4M1

Familiarity with the RIA was acquired, and although more practice was necessary, results were encouraging.

2) Part II: Pilot transplantation of the neurovascular free flap

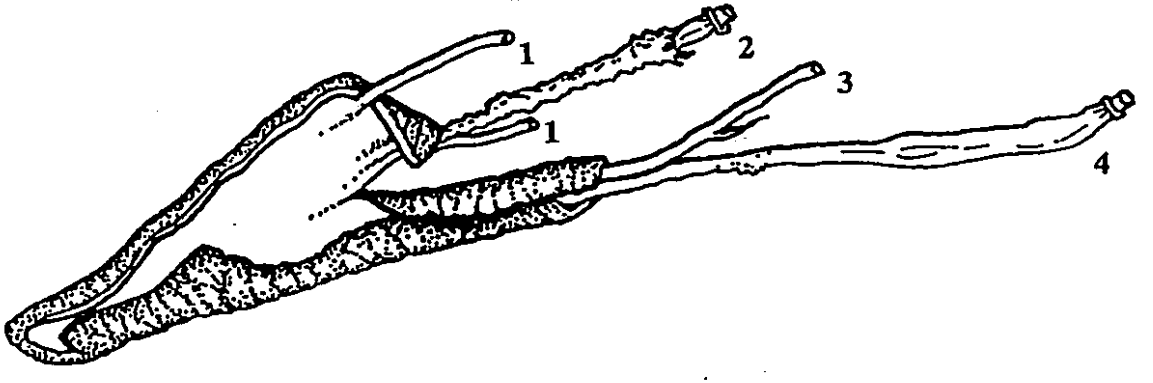
A) Technique In this part of the experiment the NVFF previously described was transplanted from an animal with a larger hand (#2) to one with a smaller hand (#3) (Table 1). The incisions were planned as described in Experiment #3 on the left second digits of both donor and recipient (81). Additionally, the volar and dorsal longitudinal incisions were extended proximally to the wrist facilitating dissection and allowing anastomosis of vessels of even larger caliber (artery 1-1.2 mm and vein 1.3-1.8 mm in diameter).

The following technique was therefore used: Under general anesthesia, the baboons were placed in the supine position with the upper limbs abducted. The proposed skin incisions were delineated, as in Experiment #3, including the addition described above. Dissection was begun in the recipient's palm and volar wrist under tourniquet control. The radial artery at the wrist was identified and gently freed from surrounding tissue. The proper palmar digital nerves were identified, isolated, labelled, and sectioned in the distal palm. The cephalic vein was located at the dorsal wrist and gently freed from surrounding tissues. Ligation and sectioning of the arterial supply to the index

finger in the distal palm and venous drainage at the distal dorsum of the hand were undertaken. The tourniquet was released, hemostasis undertaken and the operative area wrapped in moist compresses.

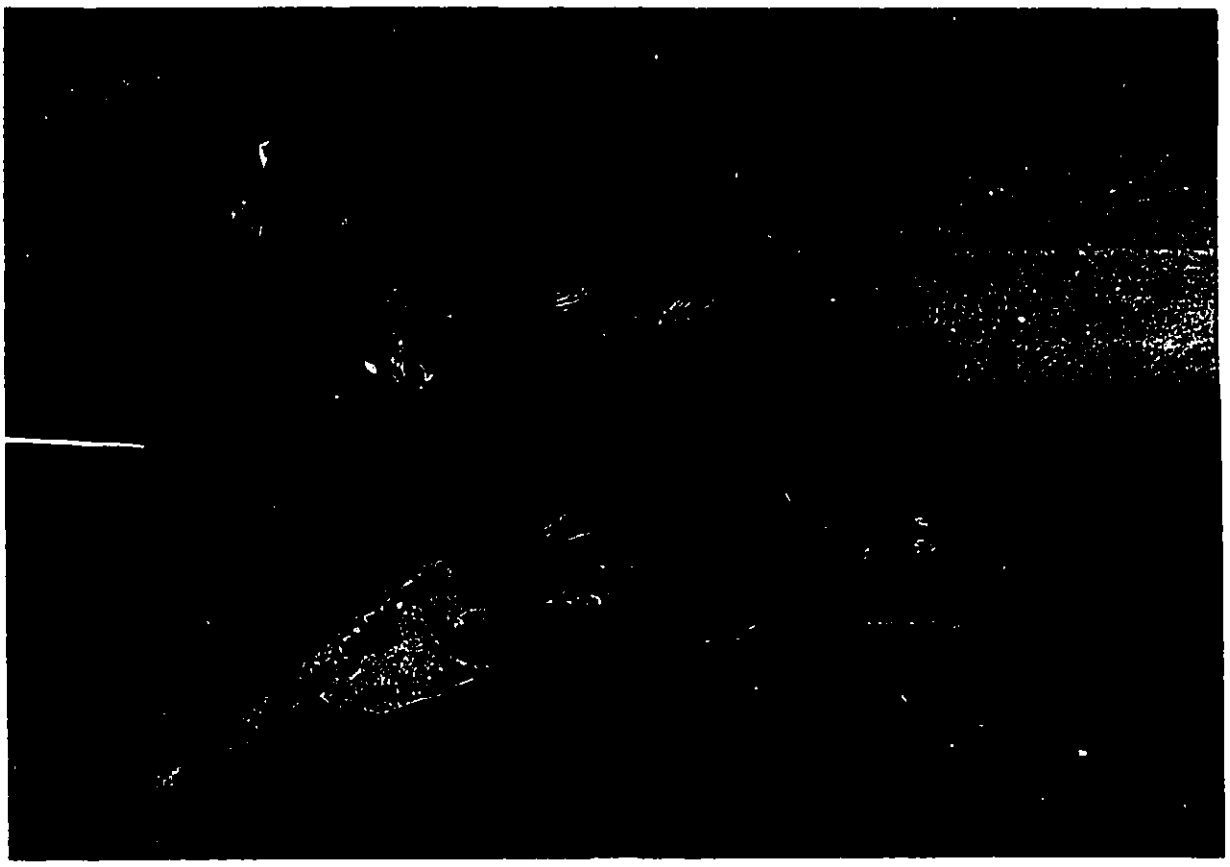
The donor flap was next prepared. Dissection was begun in the donor's palm and volar wrist under tourniquet control. The proper palmar digital artery to the ulnar side of the second digit was isolated proximally via the radial component of the volar arch to the radial artery at the wrist. All unnecessary side branches (the proper palmar digital artery to the radial side of the third digit, the ulnar side of the volar arch and the common palmar digital artery to the first web space) were ligated and sectioned. The proper palmar digital nerves were identified, isolated, labelled and sectioned in the proximal palm. The dorsal vein was located and dissected proximally to the wrist. The flap was raised superficially to the extensor apparatus and flexor tendon sheath from distal to proximal while protecting the neurovascular structures. The tourniquet was let down, hemostasis undertaken and the flap allowed to be revascularized for 10 to 15 minutes, while intravenous heparin was administered. The artery and vein were clamped with an Acland 2V Vessel Approximator in the distal forearm, sectioned and ligated just proximal to the clamps. The flap was completely removed from its bed (Figure 9) and transferred to the other baboon (Figure 10).

Figure 9: TNVFF (animal #2).



1 proper palmar digital nerve
2 radial artery
3 superficial branch of the radial nerve
4 cephalic vein

Figure 10: TNVFF (animal #2) next to recipient hand (animal #3) at transplantation. Components of the TNVFF pedicle layed out in identical fashion to those in Figure 9 (Permission granted by Little, Brown, Co. Ann. Plast. Surg. Vol. 13(5): 423-30, 1984).



The recipient's flap was excised superficially to the extensor apparatus and flexor tendon sheath from distal to proximal, and the donor flap anchored with a few sutures to its new site. Vessel diameter and length were assessed allowing the recipient radial artery and cephalic vein to be ligated and sectioned at the appropriate level for favorable microsurgical repair. Revascularization was undertaken as previously described (Experiment #3) after administration of intravenous heparin. The ends of the appropriate nerve bundles were next cut back as necessary to achieve the best size match possible as far distal in the palm as feasible. Their microsurgical repair and the rest of the procedure was undertaken as described previously (Experiment #3).

B) Intraoperative results Intraoperatively the success of the microanastomoses was shown by the patency test (62). In addition, normal color, briskness and quantity of bleeding were present from the undersurface of the flap before hemostasis and from a stab wound to the distal epidermis.

C) Postoperative results Verification of the TNVFF on postop Day 6 (day 9 of CyA administration) showed moderate swelling, mild erythema over the dorsum of the metacarpophalangeal joint (Figure 11), an area of necrosis or dehiscence of the radial distal flap, otherwise normal color, temperature, and bleeding response to a distal volar stab wound. Cyclosporin A serum trough levels were at the

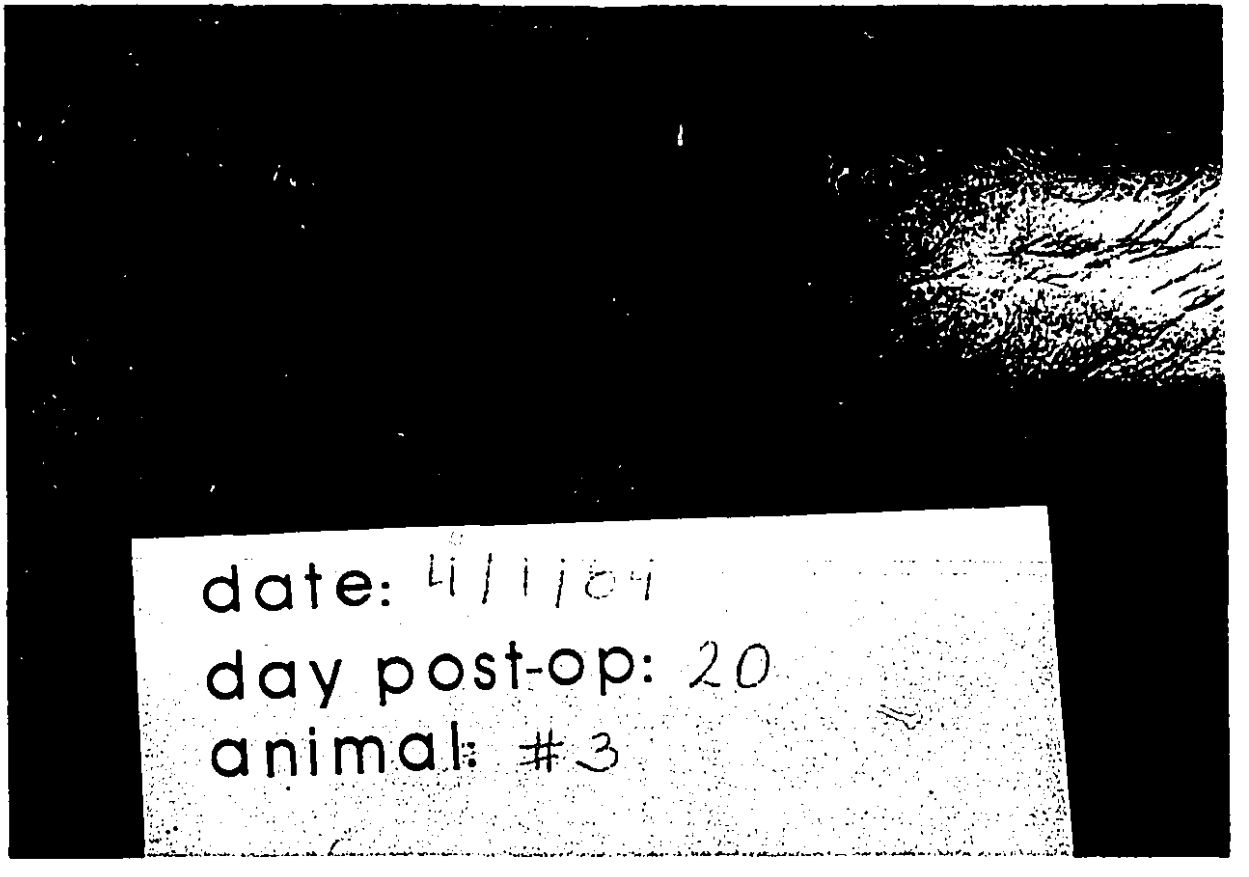
Figure 11: Dorsal TNVFF 6 days post-transplantation (animal #3). Moderate swelling and mild erythema present in the proximal TNVFF.


date:
day post-op:
animal:

upper end of the intended range. By postop Day 13 (Day 16 of CyA administration) the swelling had increased, inflammation was evident, and some desquamation was present despite CyA serum trough levels above the 200 ng/mL targeted. An infection was confirmed when Enterobacter cloacae was cultured from the flap. A biopsy taken was reported 3 weeks later and did not help identify the cause of the problem (infection versus rejection). Nevertheless, the flap continued to remain viable with a normal bleeding response to a stab wound. Despite increasing CyA dosage, switching to oral administration and treatment with an aminoglycoside, netilmycin, by postop Day 20 (Day 23 of CyA administration) no improvement was noted and despite a normal bleeding response to a distal volar stab wound (Figure 12), the experiment was terminated. Pathology revealed a necrotic flap at the suture line with findings possibly indicative of rejection.

D) Summary This first TNVFF was undertaken with the goal of perfecting all aspects of the transplantation project peri- and postoperative care protocols. These included CyA dosage, administration, and RIA (no steroids were considered in this first transplant), operative technique, infection and rejection treatment and prevention, monitoring of toxicity, analgesia, splinting, and general animal care.

Figure 12: Dorsal TNVFF 20 days post-transplantation (animal #3). Increasing swelling, inflammation, and desquamation present in the TNVFF due to rejection and infection.


date: 4/1/84
day post-op: 20
animal: #3

This transplant alerted us to several important problems: 1) we had been aiming at maintaining serum trough CyA levels below 200 ng/mL (127, 233) as suggested by Sandoz (Canada), since there was increasing opinion that this level was both sufficient and safe for renal transplant patients, 2) we had tried to administer CyA orally, as per the recommendations of Sandoz (Canada), 3) the donor animal had been estrous at the time of transplantation with several ulcers present in the peri-anal area, and 4) no prophylaxis had been given for coliforms or other bacteria found abundantly in the gastrointestinal tract and liable to contaminate the wound. Immediate implementation of the following modifications in the perioperative care plan were therefore made: 1) since skin appeared to be more immunogenic than some other organs or tissues (17, 146, 196), undergoing rejection more easily (17, 40, 136, 146, 196, 246) and to require higher CyA levels for survival (2, 246), particularly in unmatched donor-recipient pairs, serum trough levels aimed for would be in the 800 to 1000 ng/mL range with boosts to over 1000 ng/mL and higher (to around 1500 ng/mL) in response to any evidence of rejection, as long as no signs of toxicity were seen, 2) CyA would be administered intramuscularly to insure adequate intake, 3) surgery on any estrous animal would be delayed until such time as the perineal swelling had passed and lesions healed, and 4) all animals, both donors and recipients, would

receive prophylactic netilmycin (50 mg i.m. twice daily), beginning the day prior to surgery, for a period of 14 days in addition to the Penlong-S^R.

Stage III: Transplantation

Experiment #7: Transplantation of the neurovascular free flap

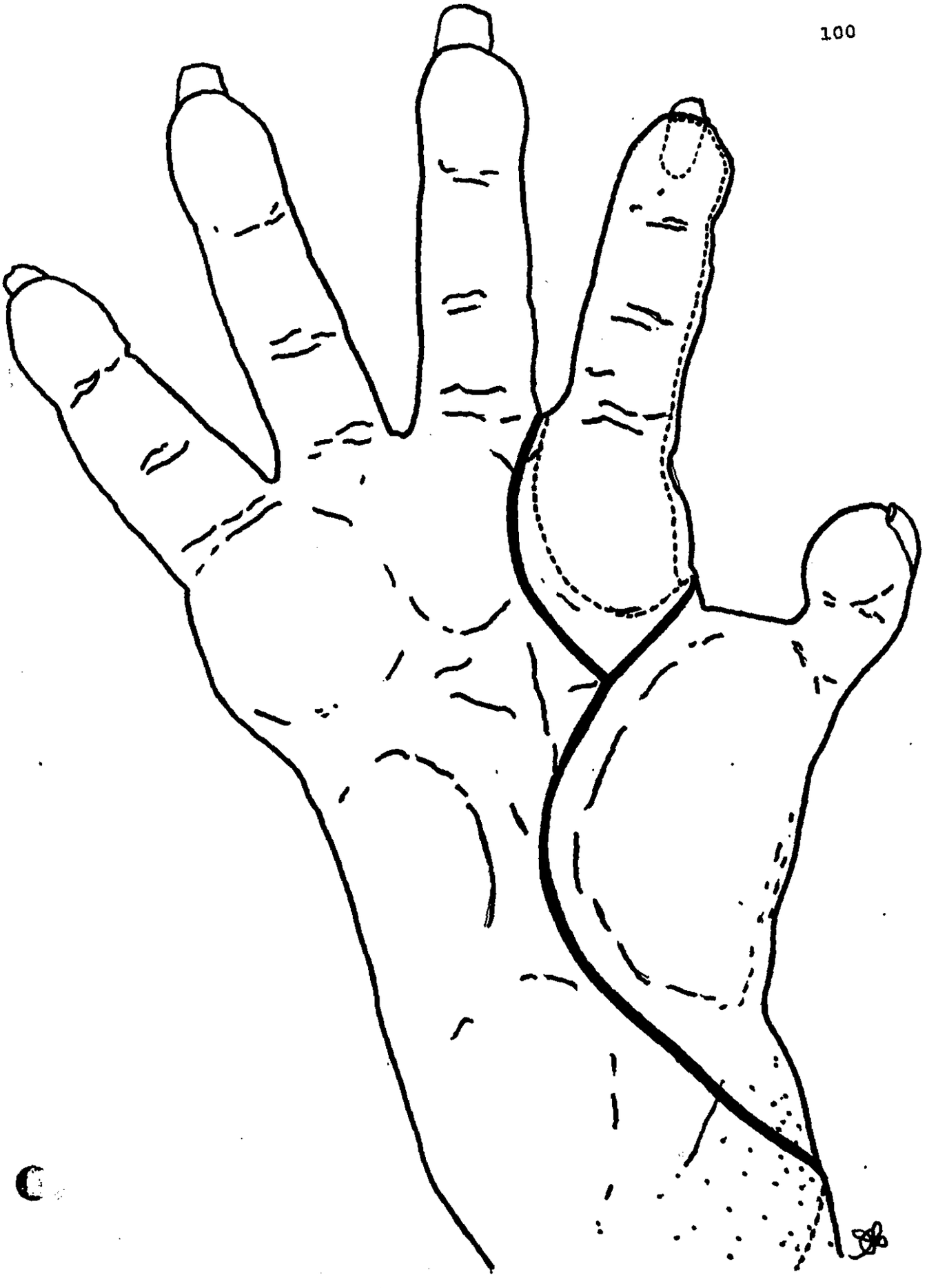
Using experience gained in Experiment #6, seven TNVFF's were undertaken (Table 4). The technique was similar to that described in Experiment #6 (58, 81) with a few modifications (Figure 13). First, upon transplanting the donor flap to the recipient bed, closure of the radial longitudinal digital incision was completed prior to revascularization to avoid the edema and to facilitate closure. The dorsal longitudinal incision in the recipient hand was eliminated so that a suture line would not lie over the length of the donor vein. To allow access to the cephalic vein at the wrist in the recipient, the volar longitudinal incision was curved dorsally upon reaching the wrist. The dorsal skin between the second digit and cephalic vein was then undermined to tunnel the flap's vein proximally to the wrist for microanastomosis. In several flaps, not only were the two digital nerves repaired, but if branches to the volar interdigital pad or proximal dorsal skin were identified in both donor and recipient, these too

Table 4: Summary of TNVFFs in Experiment #7

Baboon	Date of Surgery	Type of Intervention	Transplant Survival	Animal Survival	Rejection	Problems
#4	23/02/84	Donor L to #	-	-	-	-
#5	23/02/84	Recipient L from #4	D161	D161 (euthanasia for renal failure)	D12 moderate (reversed) D96 mild to moderate (reversed)	loss autogenous nail L index, wound dorsal PIP joint in TNVFF requiring autogenous skin graft, transient decreases in Hb, intermittent anorexia, gingival hyperplasia requiring gingivectomy, transient then irreversible renal failure
#10	17/05/84	Donor R to #11	-	-	-	-
#11	17/05/84	Recipient R from #10	D413 (amputation)	D532 (euthanasia)	D18 possible mild persistent D106 moderate fluctuating persistent	intermittent anorexia, gingival hyperplasia, transient decrease in WBC and Hb, abscesses, edema limbs
#14	13/07/84	Donor R to #15	-	-	-	-
#15	13/07/84	Recipient R from #14	D147 (amputation)	D149 (euthanasia)	D61 moderate to severe (reversed) D75 mild, fluctuating persistent, gradually more severe	anorexia
#16	11/09/84 08/10/84	Donor R to #17 Donor L to #20	- -	- -	- -	- -
#17	11/09/84	Recipient R from #16	D141	D141 (euthanasia for lymphoma)	nil	gingival hyperplasia, intermittent anorexia, falling Hb, edema lower limbs multiple lymph nodes, splenomegaly, decreased platelets
#18	26/10/84 23/11/84	Donor R to #21 Donor L to #19	- -	- -	- -	- -
#19	23/11/84	Recipient L from #18	D122 (D1237) (last dressing change)	D123 (died with severe anemia)	D38 mild to moderate fluctuating (reversed) D118 mild to moderate persistent	transient anorexia, falling Hb, abscesses
#20	08/10/84	Recipient L from #16	D193 (amputation)	D219 (euthanasia)	nil	gingival hyperplasia requiring gingivectomy, transient decrease in Hb, anorexia
#21	26/10/84	Recipient R from #18	D196 (amputation)	D213 (euthanasia)	D26 very mild (reversed)	gingival hyperplasia, transient anorexia, abscesses, transient decrease in Hb, edema thighs

D : postoperative day
PIP: proximal interphalangeal
Hb : hemoglobin
WBC: white blood cell count

Figure 13: Design for the TNVFF (animals after #3).



were microanastomosed. Beginning with the second transplant, the fingertip, including the nail of the recipient, was amputated to allow complete primary closure of the area using the donor flap, thus avoiding any poorly vascularized tissue (a problem in baboon #5).

Animal care, although generally undertaken as described in the Materials and Methods section, did undergo some fine tuning before reaching its final form as we progressed along the learning curve and we occasionally encountered difficulties with individual animals (e.g., missing a day of acetylsalicylic acid administration because of animal refusal), forcing us to deviate from our intended course. A few specific points of importance should nevertheless be mentioned.

Cyclosporin A: Although the first animal in the group (#5) received only three preoperative oral doses of CyA at an average dose of 15.6 mg/kg, by the time the last baboon (#19) TNVFF was undertaken this had been modified to seven preoperative intramuscular doses of 26.6 mg/kg CyA each. In between, most animals received seven preoperative intramuscular administrations of CyA at an average dose of 22.6 mg. This increase in preoperative CyA treatment was undertaken in response to two factors. First, in the first transplant (#5), it quickly became evident that these transplants presented such a strong immunogenic stimulus that the recipient would respond vigorously if significant

immunosuppression was not established early. Second, as we gradually became familiar with the pharmacologic characteristics of the CyA in its hydrophobic vehicle, we noted that several days of administration were required before serum levels would begin rising towards the therapeutic range.

It should also be mentioned that another vehicle for CyA administration was tried in three (#11,#15,#19) of the seven animals for up to 6 weeks. Cyclosporin A dissolved in Cremaphor-EL[22] was administered subcutaneously, requiring lower dosages than the intramuscular preparation (209). Unfortunately, this product caused serious skin ulceration in some animals with its extended use (#11,#19) forcing us to return to the original intramuscular preparation.

In one baboon (#19) oral dosing was once again attempted with the Miglyol 812 preparation. The same problems recurred as with previous attempts, the animal discarding significant portions of each dose. The medication's hydrophobic nature and its disagreeable flavor made its incorporation into palatable foods almost impossible to administer. Adequate levels could therefore not be maintained.

Steroids: Steroids were only administered in response to histologic evidence of rejection in the first transplant

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[22]Sandoz, Ltd., CH-4002, Basle, Switzerland

(#5). The report from the biopsy made on Day 12 was available 3 days later and indicated the presence of chronic and acute inflammation. In reaction to the histologic suggestion of rejection, the previously established protocol was implemented on Day 15, 3 full days after clinical suspicion (the latter based on the presence of excessive swelling, erythema, maceration and epidermal sloughing). A biopsy taken on Day 23 and reported 5 days later indicated that the inflammation had improved but was still present in the upper dermis while the presence of necrotic cells had decreased. Yet, by Day 27, the day before the histology result became available, clinical evidence of improvement was already visible (swelling gone, flap dry).

In the remaining TNVFFs it was decided that the rejection protocol would be implemented once clinical signs of rejection were evident rather than awaiting the results from the biopsy specimens. In animal #11 steroids were therefore started on Day 3. Despite early concerns of infection which were not borne out, it soon became clear that in the remaining transplants, steroids would have to become a standard part of the immunosuppression protocol, as it is in some clinical protocols (13, 25, 29, 102, 127, 165, 215, 216, 217, 230), the first dose being given at surgery. Nevertheless, it took further experience to recognize the slightest changes suggestive of rejection and to appreciate the importance of immediate action.

1) Intraoperative results Intraoperatively, the success of the microanastomoses was proven in all transplanted flaps by the patency test (62). In addition, normal color, briskness, and quantity of bleeding were present in all cases from the undersurface of each flap before hemostasis and from a stab wound to the distal epidermis at the end of surgery.

2) Postoperative results

A) Technical assessment Postoperative evaluation of each TNVFF's viability was undertaken twice in the first week, beginning on Day 3 (except for the first transplant, #5, in which initial postoperative verification was on Day 6). All flaps showed evidence of good circulation based on assessment of color, turgor, temperature, and bleeding response to an epidermal stab wound. No flap was lost due to technical failure of the microanastomoses.

B) Survival times Survival times for the TNVFF ranged from 122 (when the dressing was last changed or possibly 123 days when the animal died) to 413 days postoperatively (Table 4). Average survival time was 196 days. It should be clarified that none of the TNVFFs ever reached the point of flap loss from rejection because all (except #17,#19) underwent neurophysiologic recording by our colleagues to evaluate reinnervation (58, 81, 199) before severe rejection could affect results. In animal #17 euthanasia was felt to be humane to prevent suffering when

signs of lymphoma became evident. In baboon #19, death occurred after trying to reinstitute oral CyA administration when hemoglobin concentration plummeted to 15 g/L. Therefore, it can be proposed that longer survival times might be possible, although likely at the expense of significant side effects.

C) Rejection Only 2/7 TNVFFs (#17,#20) showed no evidence of rejection (Figures 14 and 15), and another (#21) underwent a very mild easily reversed episode (Figures 16 and 17) (Table 4). All other allografts showed significant clinical evidence of rejection at some time after transplantation. Mild signs included: edema, erythema and sloughing of a thick, firm, epidermal layer revealing healthy, intact epidermis (Figure 18). Moderate signs, in addition to the above, included weeping from small areas of breakdown to raw, red dermis (Figure 19), and in chronic cases, persistent flaking was present (Figure 20). With severe rejection, large areas of epidermal slough were present, leaving behind plaques of angry, red, moist dermis (Figure 21), occasionally covered with a layer of whitish-green infected exudate. In all cases of rejection, pigmentation was at least temporarily affected and localized hair loss correlated roughly with severity.

It was our subjective impression that, once rejection had occurred in a TNVFF, stability was more difficult to achieve and subsequent rejection episodes were difficult to

Figure 14: TNVFF 141 days post-transplantation (animal #17).
No evidence of rejection.

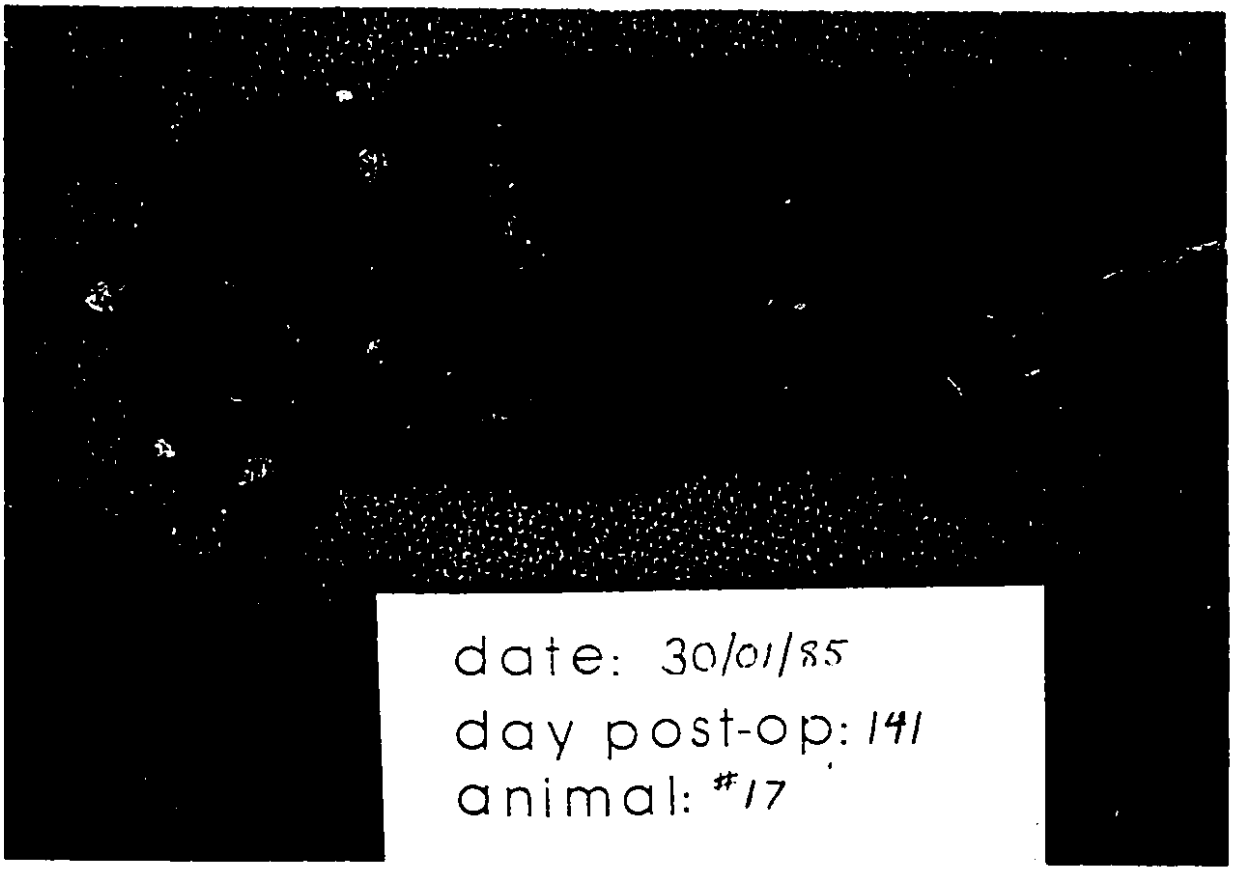

date: 30/01/85
day post-op: 141
animal: #17

Figure 15: TNVFF 190 days post-transplantation (animal #20). No evidence of rejection.

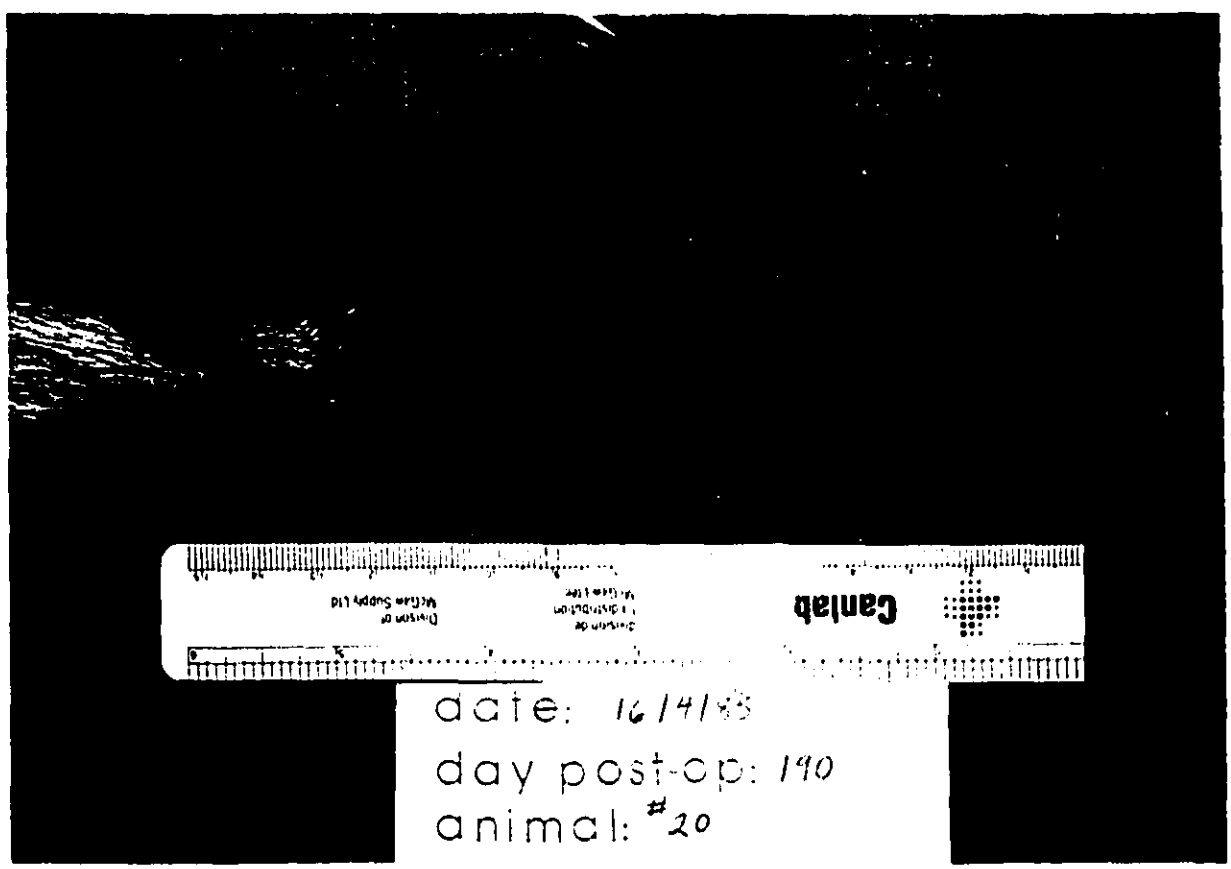

Canlab
date: 16/4/93
day post-op: 190
animal: #20

Figure 16: TNVFF 32 days post-transplantation (animal #21). Very mild rejection with edema and thickening of the epidermal layer.

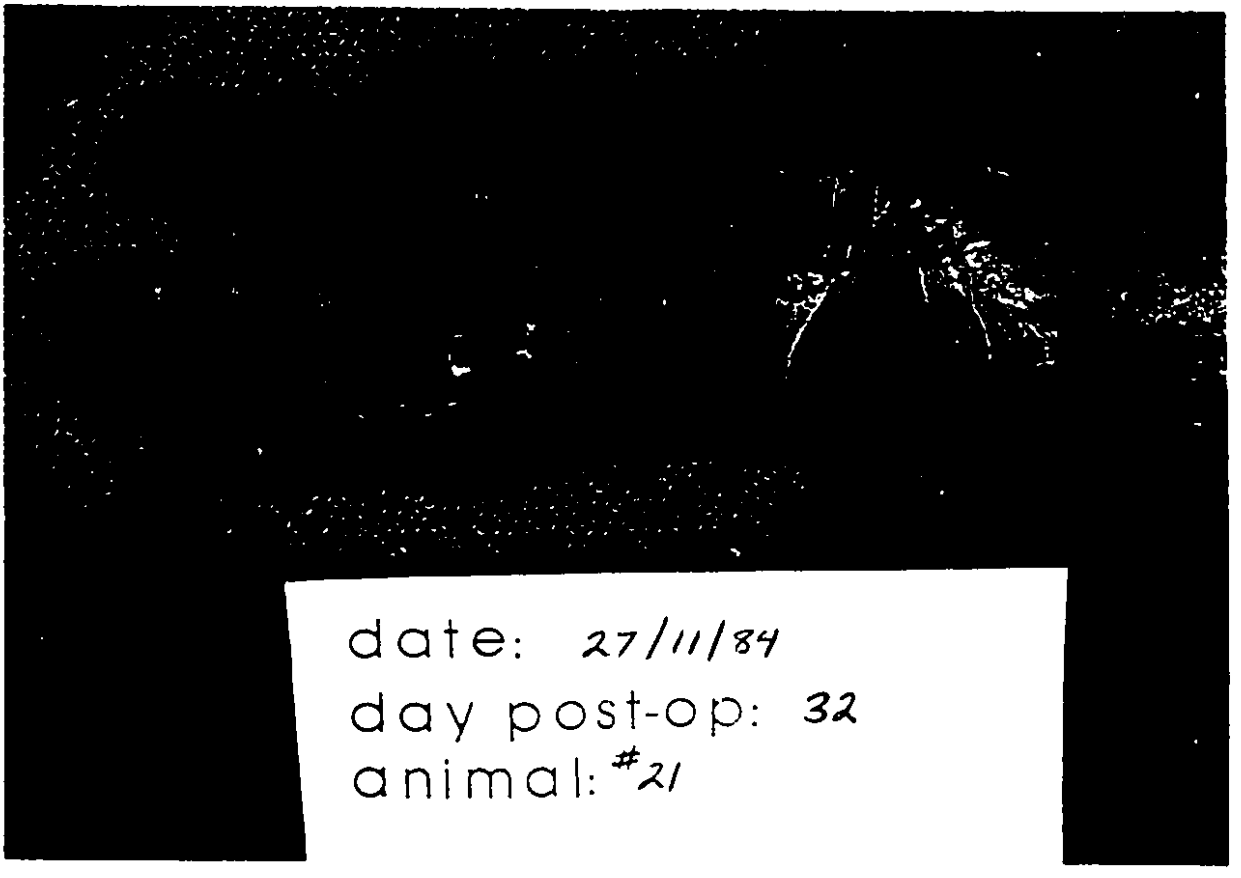

date: 27/11/84
day post-op: 32
animal: #21

Figure 17: TNVFF 193 days post-transplantation (animal #21). The hand is oriented differently from Figure 16, but is the right hand of the same animal 161 days later. No evidence of rejection.

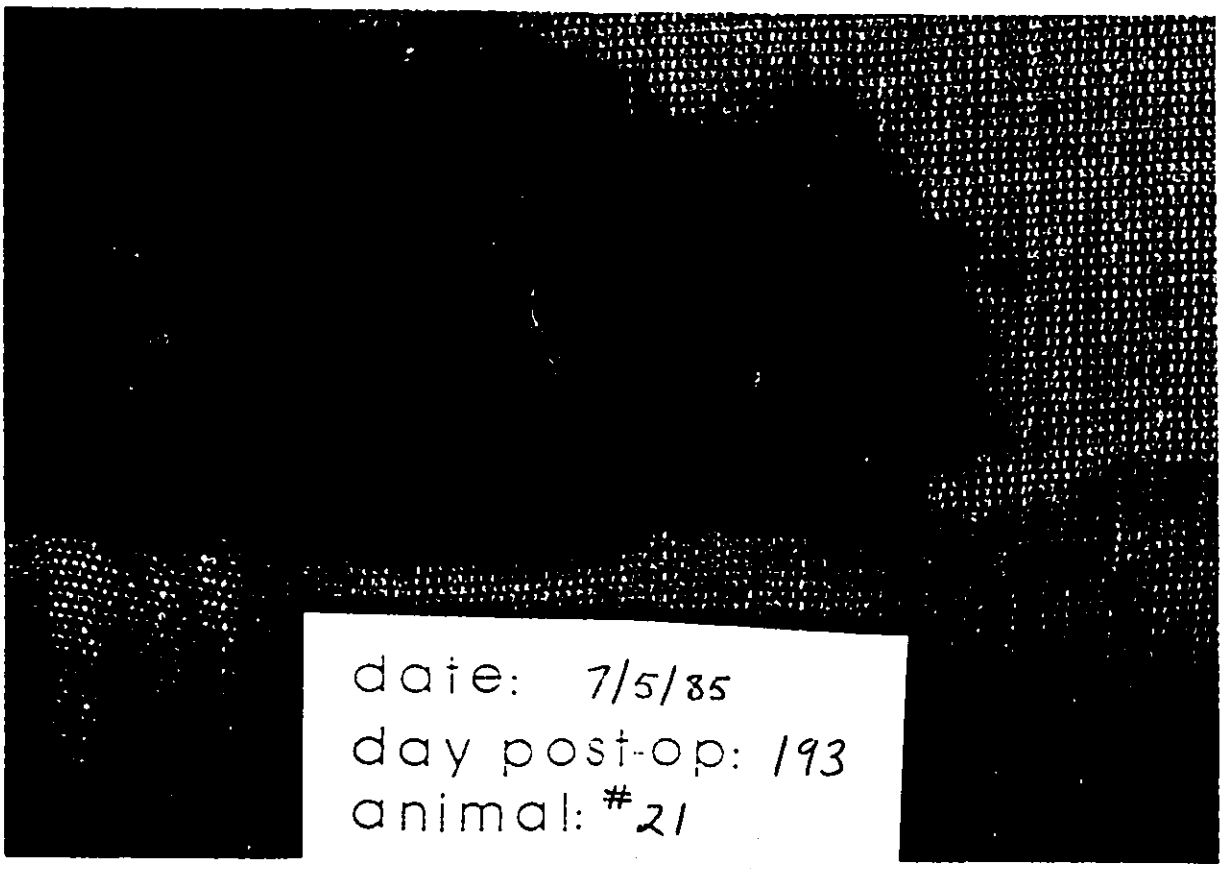
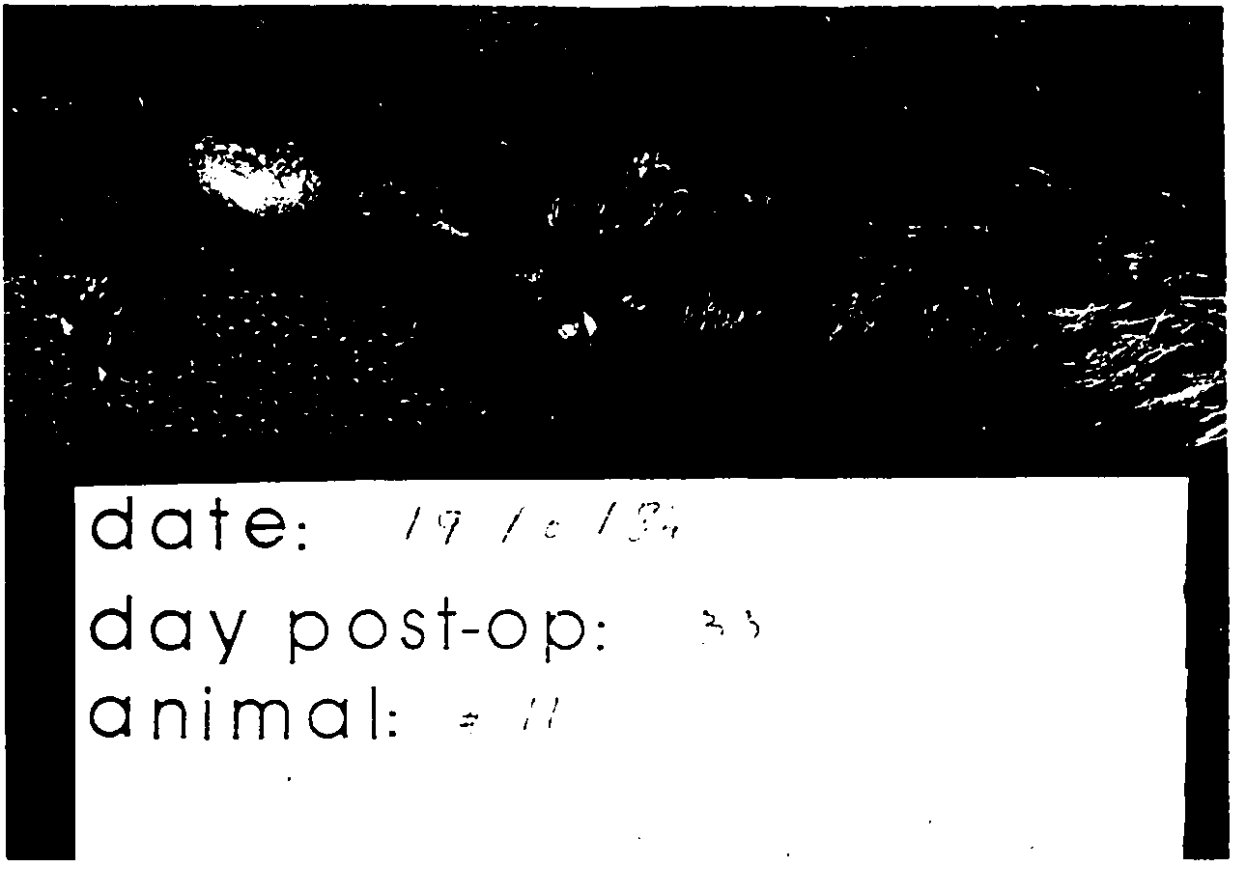

date: 7/5/85
day post-op: 193
animal: #21

Figure 18: TNVFF 33 days post-transplantation (animal #11). Mild rejection with edema and sloughing of a thick, firm, epidermal layer revealing healthy, intact epidermis.



date: 19/6/94

day post-op: 33

animal: # 11

Figure 19: TNVFF 124 days post-transplantation (animal #11). Moderate rejection including weeping from small areas of breakdown to raw, red dermis.

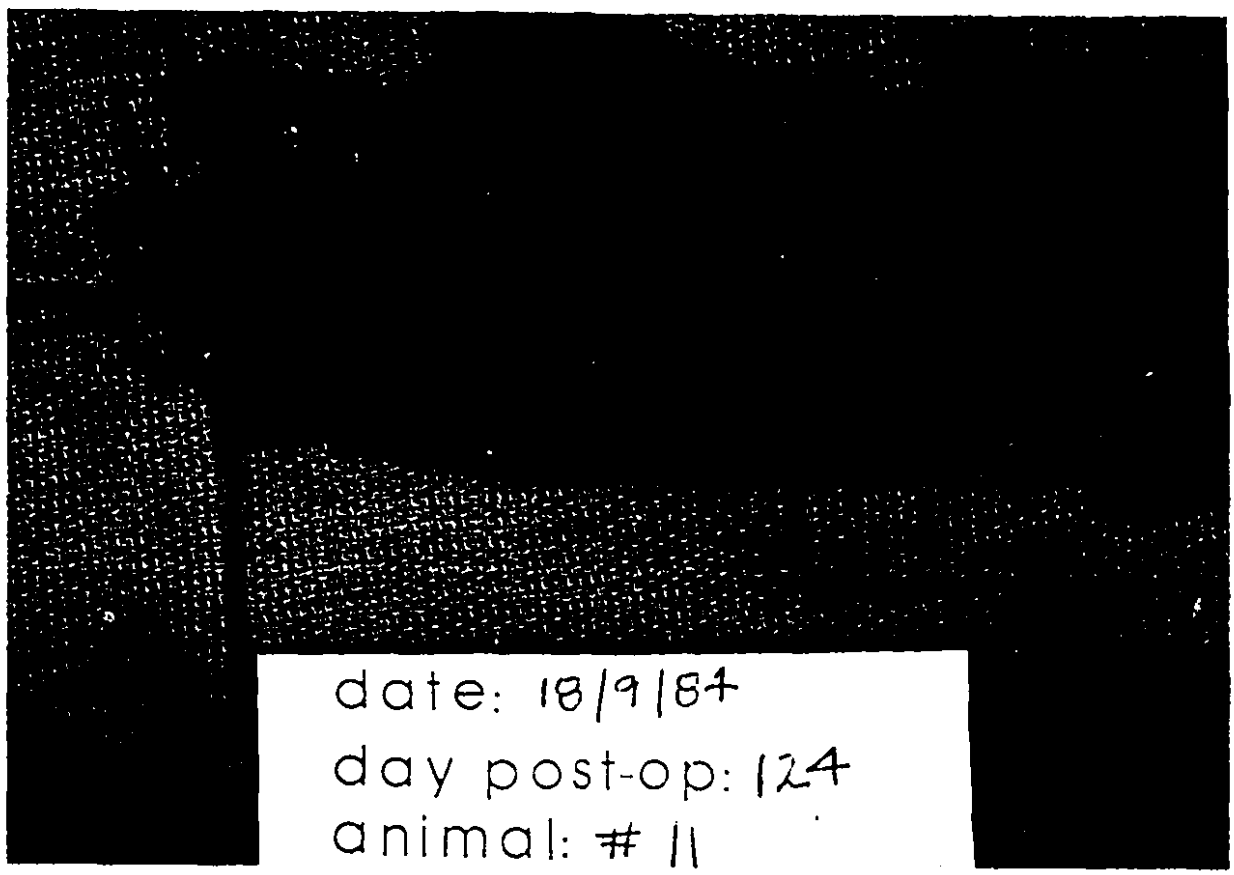

date: 18/9/84
day post-op: 124
animal: # 11

Figure 20: TNVFF 277 days post-transplantation (animal #11). Moderate chronic rejection with persistent flaking.

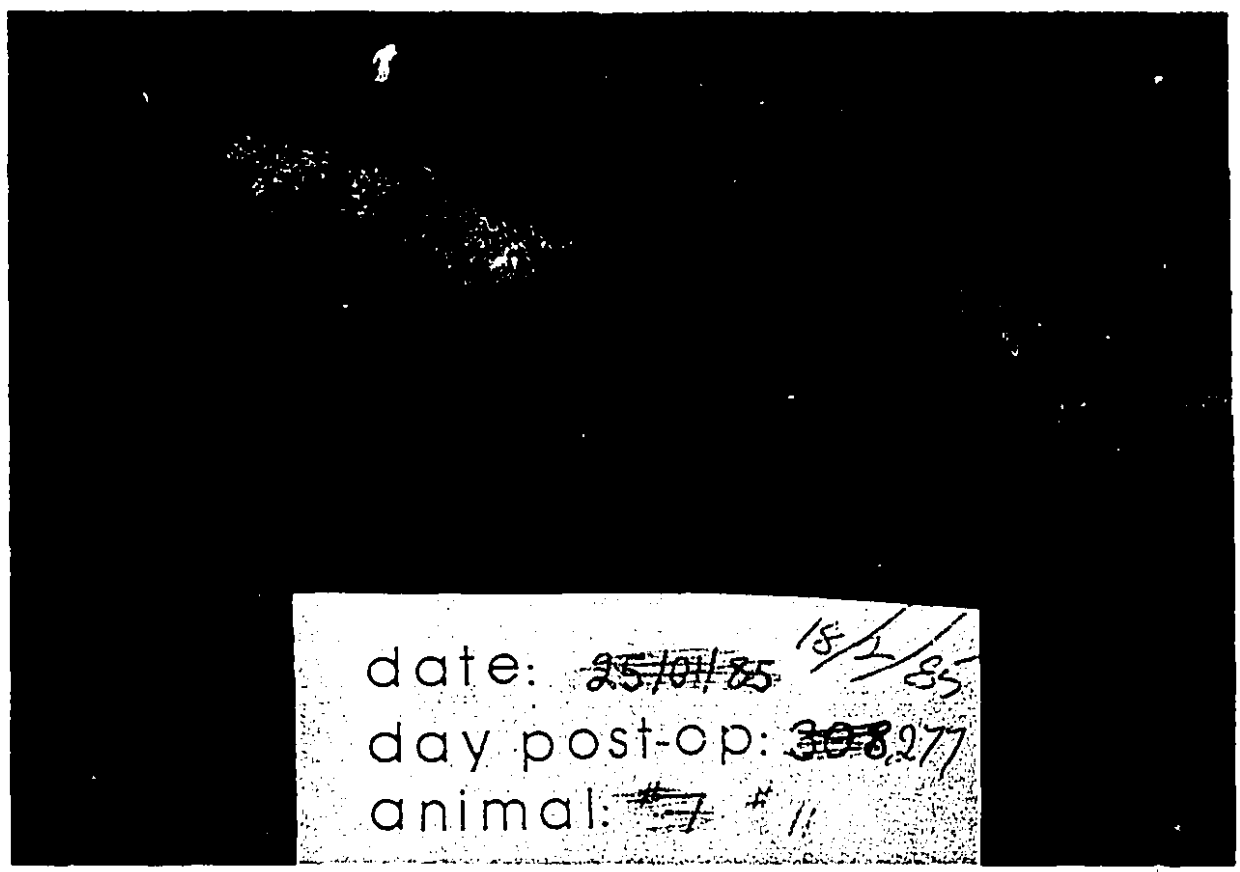
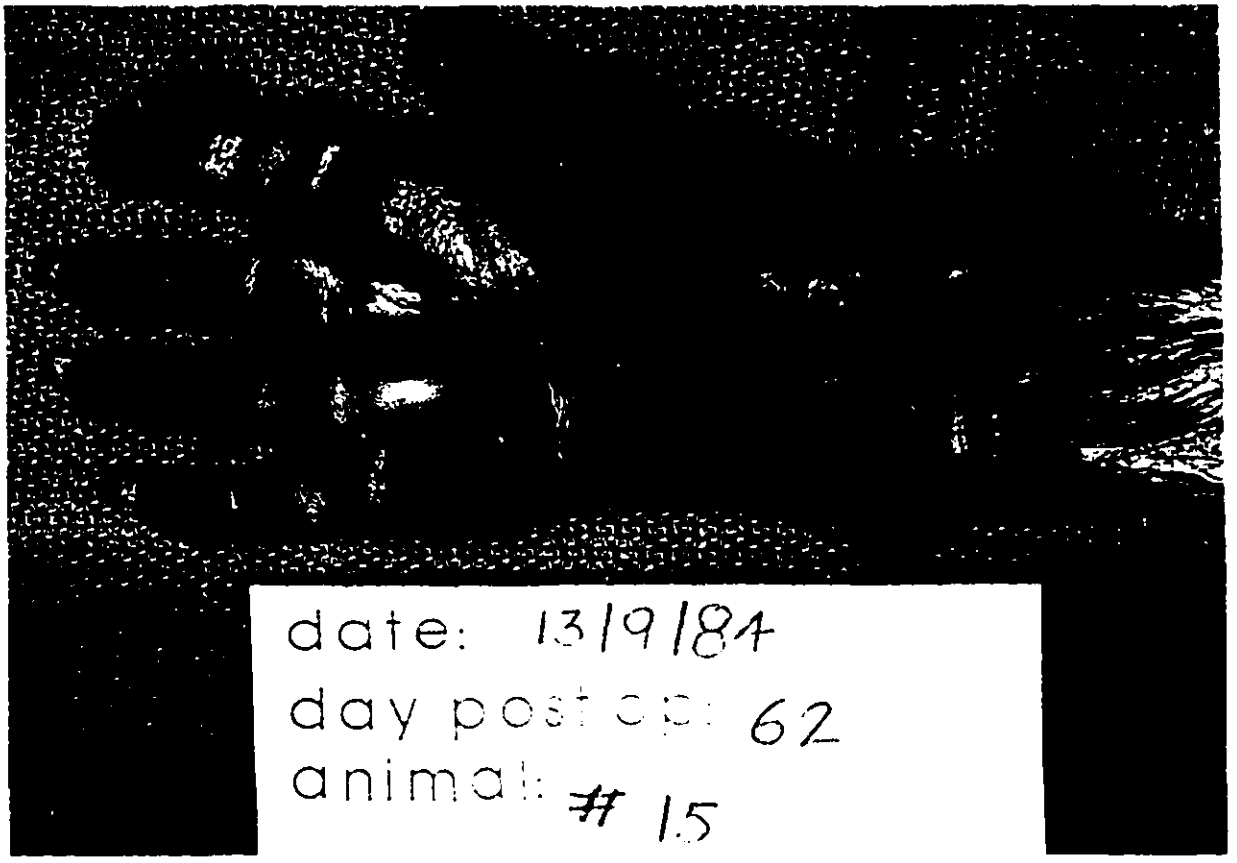

date: ~~25/01/85~~ 15/1/85
day post-op: ~~308~~ 277
animal: ~~#7~~ # 11

Figure 21: TNVFF 62 days post-transplantation (animal #15). Severe rejection with large areas of epidermal slough leaving plaques of angry, red, moist dermis.


date: 13/9/84
day post op: 62
animal: # 15

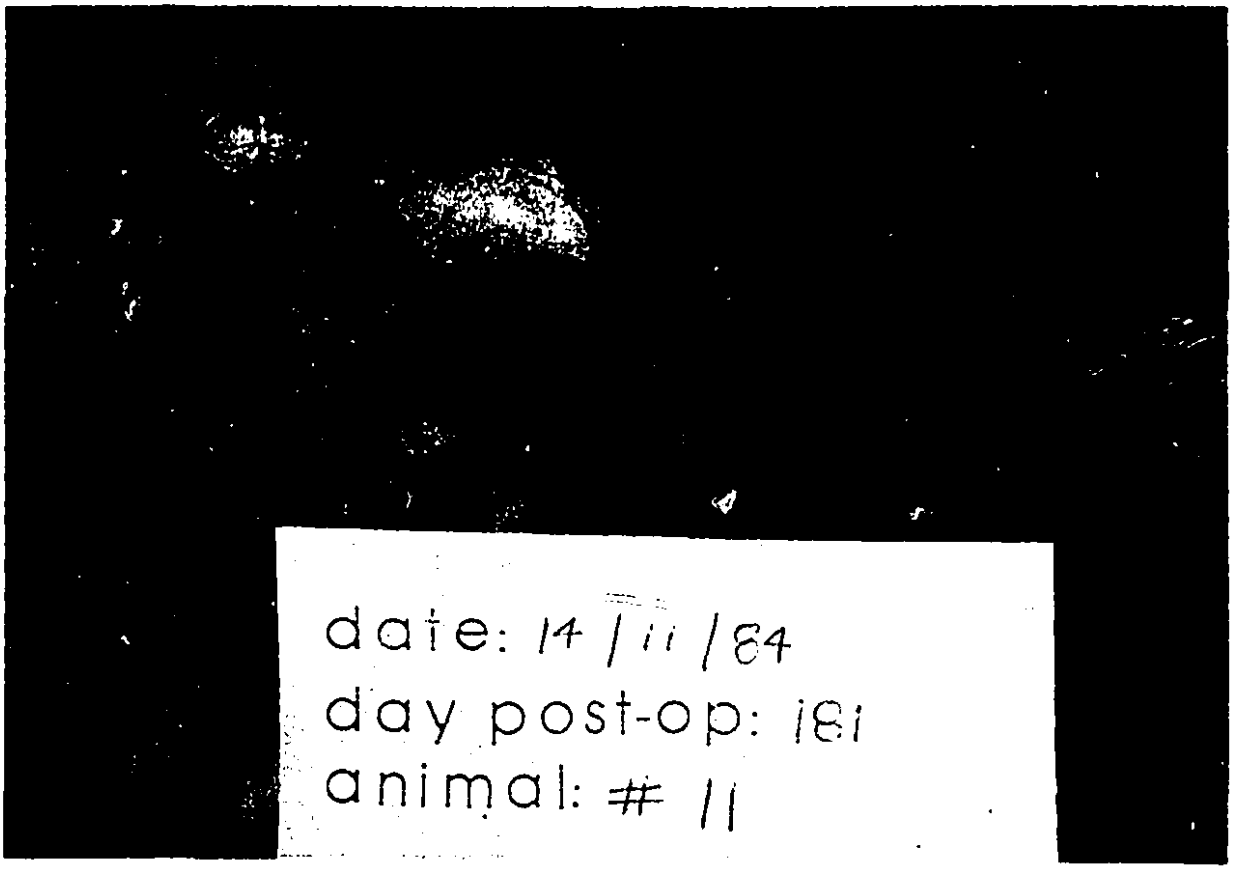
avoid. Although this observation is difficult to prove with the data from this study, it may be related to sensitization taking place during the first rejection episode. Subsequent episodes may therefore act more like second set rejection reactions, since the immune system would be primed and ready to respond along multiple fronts.

Despite the many rejection episodes, the rejection protocol was proven to be effective in at least controlling (Figures 19 and 22) and even reversing the process (Figures 16 and 17). As experience was gained, the protocol was put into effect in response to increasingly milder signs of trouble with significant improvement in results.

3) Summary Since the most successful transplants all occurred in the latter part of the learning curve, experience probably contributed to a more stable course. It is therefore possible that further studies might have allowed continued improvement.

Three additional factors, which likely affected the overall success of this experiment, deserve mention. First, the daily care of the animals was transferred during the course of the project. Despite trying to transmit all the accumulated experience, it was impossible to avoid the establishment of a new learning curve with its associated consequences. Second, the subcutaneous use of the Cremaphor-EL CyA seemed consistently related to the occurrence of a rejection crisis after several weeks of its

Figure 22: TNVFF 181 days post-transplantation (animal #11). Moderate rejection controlled using the rejection protocol.


date: 14 / 11 / 84
day post-op: 181
animal: # 11

use. None of the three most successful transplants (i.e., with little or no rejection episodes) had received this compound. Third, during the course of this experiment, there was a period during which the CyA RIA was difficult to interpret and modifications were being tried to improve accuracy. One of these modifications, warming the sample to body temperature during measurement, made results appear approximately 200 ng/mL higher than usual (75, 140). Without concomitant modification of the acceptable therapeutic range, levels were inadvertently allowed to slip below the lower limit of the accepted window.

Despite these problems, several key points for success in this model were established. Cyclosporin A in the baboon appears to be most dependably administered in a Miglyol 812 vehicle by twice daily intramuscular injections (73). This CyA mixture must be started several days preoperatively (4 days seems to be appropriate) to allow its levels to rise sufficiently for uneventful transplantation. Steroids are required in addition to the CyA, as used by some clinical centers (13, 25, 29, 102, 127, 165, 215, 216, 217, 230), for sufficient immunosuppression to be established in this model. Cyclosporin A levels required appear to fall within the 800-1500 ng/mL range by RIA (the author would aim for 1000 ng/mL) when the sample is at room temperature. Immediate response to even the slightest signs of rejection with the rejection protocol permit the smoothest rejection

free course. Once a rejection episode occurs, subsequent episodes are difficult to prevent. The rejection protocol is effective in controlling and even reversing rejection episodes.

All TNVFFs survived to or beyond the 4 months it normally takes for reinnervation of the distal tip of the fifth digit following transection of the ulnar nerve at the wrist (229). This model has therefore fulfilled the aims set out in the beginning, although further refinements could be useful.

Experiment #8: Hand transplantation

Using experience gained in Experiments #4, #6 and the early part of Experiment #7, four hand transplants (hereafter HT) were undertaken (Table 5). The technique was similar to that described in Experiment #4 with a few modifications (Figure 23) (58, 81). First, dissection was completed in the recipient before undertaking the donor. Once the flexor and extensor tendons were identified, they were tagged, marked with a suture 2 cm proximal to the wrist and sectioned at the joint in the recipient and as far proximal as possible in the donor (Figure 24). As the median, ulnar and radial nerves were identified and tagged, they were sectioned approximately 2 cm proximal to the wrist in the first animal and at a level to retain maximal length with the donor segment in the second. Transection of the

Table 5: Summary of HTs in Experiment #8

Baboon	Date of Surgery	Type of Intervention	Transplant Survival	Animal	Rejection Survival	Problems
#6	23/03/84	Donor R to #7	-	-	-	-
#7	23/03/84	Recipient R from #6	D311	D311 (euthanasia)	D5 mild (reversed) D140 mild to moderate (reversed) D186 mild to moderate fluctuating persistent	intermittant anorexia, R elbow/forearm/wrist stiffness, gingival hyperplasia
#8	04/05/84	Donor L to #9	-	-	-	-
#9	04/05/84	Recipient L from #8	D26	D26 (euthanasia)	DO hyperacute	anorexia
#12	05/07/84	Donor L to #13 Recipient L from #13	D69 (D717) (last dressing change)	D71 (euthanasia)	D3 mild to moderate (reversed) D48 severe fluctuating persistent	severe anorexia and dehydration
#13	05/07/84	Donor L to #12 Recipient L from #12	D188 (amputation)	D208 (euthanasia)	D6 mild to moderate (reversed) D55 severe controlled, then mild to moderate fluctuating persistent	early bulse forearm, decreased hair growth forearm than on HT, transient atrial fibrilation, intermittent anorexia

D : postoperative day

Figure 23: Schematic representation of surgical technique for HTs (Permission granted by Mosby-Year Book, Inc., J. Hand Surg. 11A(1): 1-8, 1986).

Vessel anastomoses
Flexors
Extensors
Nerve anastomoses
Bone shortening
2cm

Figure 24: Donor HT (animal #8) to animal #9 at transplantation prior to amputation. Neurovascular elements and tendons are identified, tagged, and the tendons marked with a suture 2 cm proximal to the wrist. In the donor, all these structures are sectioned as far proximally as possible while the radius and ulna are transected 4 cm proximal to the wrist.

radius and ulna 4 cm proximal to the wrist preceded ligation of the vascular pedicle in the donor whereas it followed the same in the recipient, only at the 6 cm mark. The radial artery and cephalic vein, once dissected, were hemoclipped and sectioned in the recipient. After allowing a 15 minute period of revascularization of the donor hand and the administration of heparin to both animals, the vessels in the donor were ligated with small hemoclips and sectioned. Vascular transection in each animal was at the same level as for the nerves, respectively (Figure 25).

Next, transplantation was undertaken beginning with bony internal fixation and repair of the deep tendons using the same technique as in Experiment #4. Tension for all tendon repairs (Figures 26 and 27) was adjusted by lining up the 2 cm marks on appropriate donor and recipient tendon pairs prior to tenorrhaphy. Following excision of redundancy, the microvascular anastomoses were undertaken using 10 to 12 simple interrupted stitches in a similar fashion to that used in Experiment #3. Once the hand was revascularized, the appropriate nerve endings were repaired using three to six simple interrupted stitches in a manner resembling that in Experiment #3. The remainder of the operation was as described in Experiment #4.

A further modification of technique was necessary for the "switch" transplant (where each animal was both donor and recipient of a left hand) between animals #12 and #13

Figure 25: Donor HT (animal #6) to animal #7 at transplantation. At the top is the amputated recipient hand (animal #7). In the middle is the recipient forearm (animal #7) with the neurovascular elements and tendons identified, tagged and the tendons marked with a suture 2 cm proximal to the wrist. In the recipient the tendons are sectioned at the joint and the neurovascular structures 2 cm proximal to the wrist. At the bottom is the amputated, prepared donor hand (animal #6). Permission granted by Little, Brown, Co., Ann. Plast. Surg. 13(5): 423-30, 1984.

Figure 26: HT at completion of transplantation (animal #12). Accurate tension adjustment at tenorrhaphy provides a tenodesis effect with finger flexion during wrist extension.

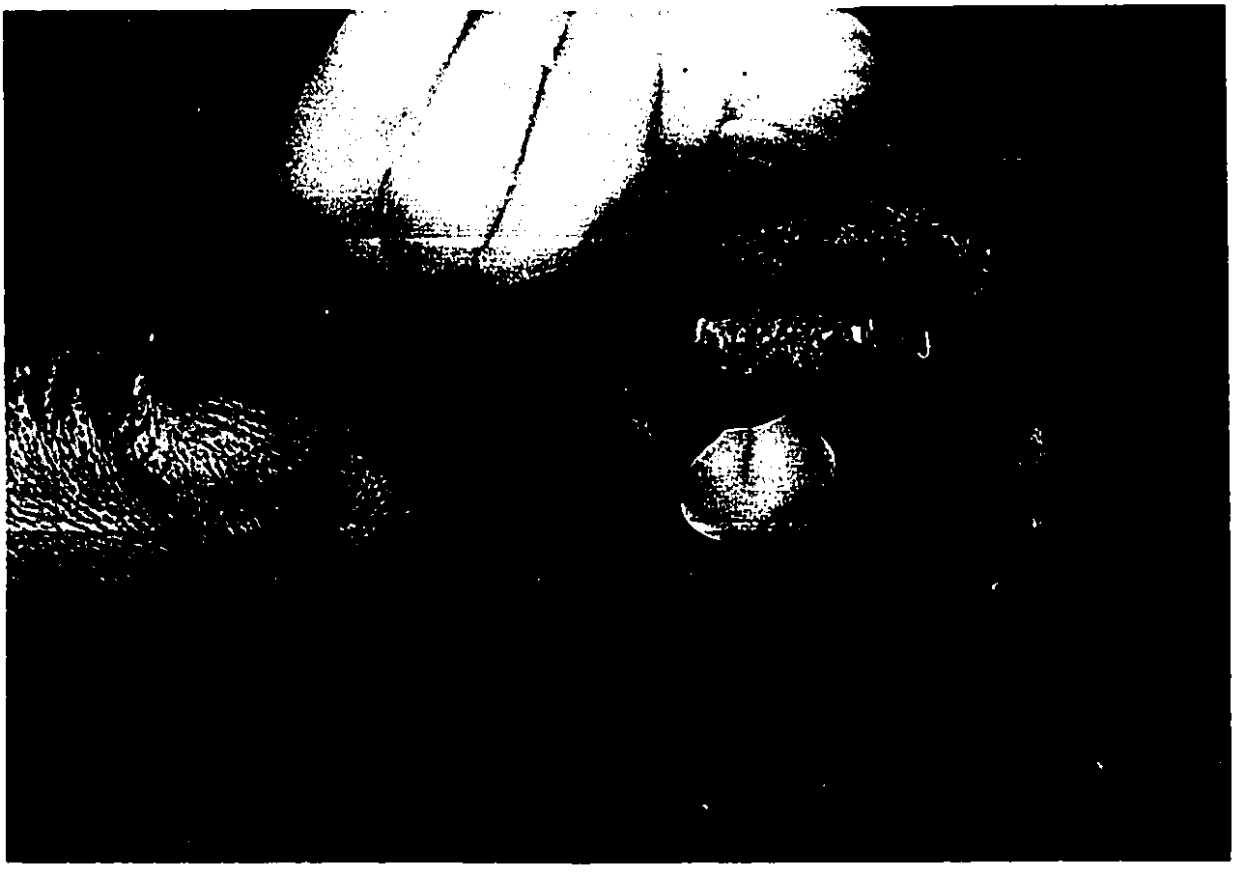


Figure 27: HT at completion of transplantation (animal #12). Accurate tension adjustment at tenorraphy provides a tenodesis effect with finger extension during wrist flexion.

(Figures 28, 29 and 30). Skin, tendons and neurovascular structures were all sectioned 4 cm proximal to the wrist in both animals. The radius and ulna were sectioned at 2 and 5 cm proximal to the wrist in both baboons, discarding the intervening 3 cm of bone to allow adequate repair of all structures while avoiding excessive tension. During the actual transplantation, two teams worked simultaneously to prevent unacceptably long ischemia times for each HT.

Animal care, as in Experiment #7, presented occasional problems and underwent significant modifications and improvements eventually contributing to the format found in the Materials and Methods section. Since these transplants were all undertaken in the early stages of our experience, similar points to those made in Experiment #7 should be highlighted.

Cyclosporin A: Although the first animal in this group (#7) received only two preoperative daily intramuscular doses of CyA at 23.7 mg/kg each, the last two received seven doses (23.1 mg/kg/dose) administered twice daily. Although the dosage was increased by the time we undertook the last TNVFF (#21), we realized early on the importance of adequate preoperative CyA priming using the intramuscular preparation, particularly in view of the greater immunogenic load of the HTs.

As in Experiment #7, the Cremaphor-EL CyA preparation was administered for up to 6 weeks in each of the HT's

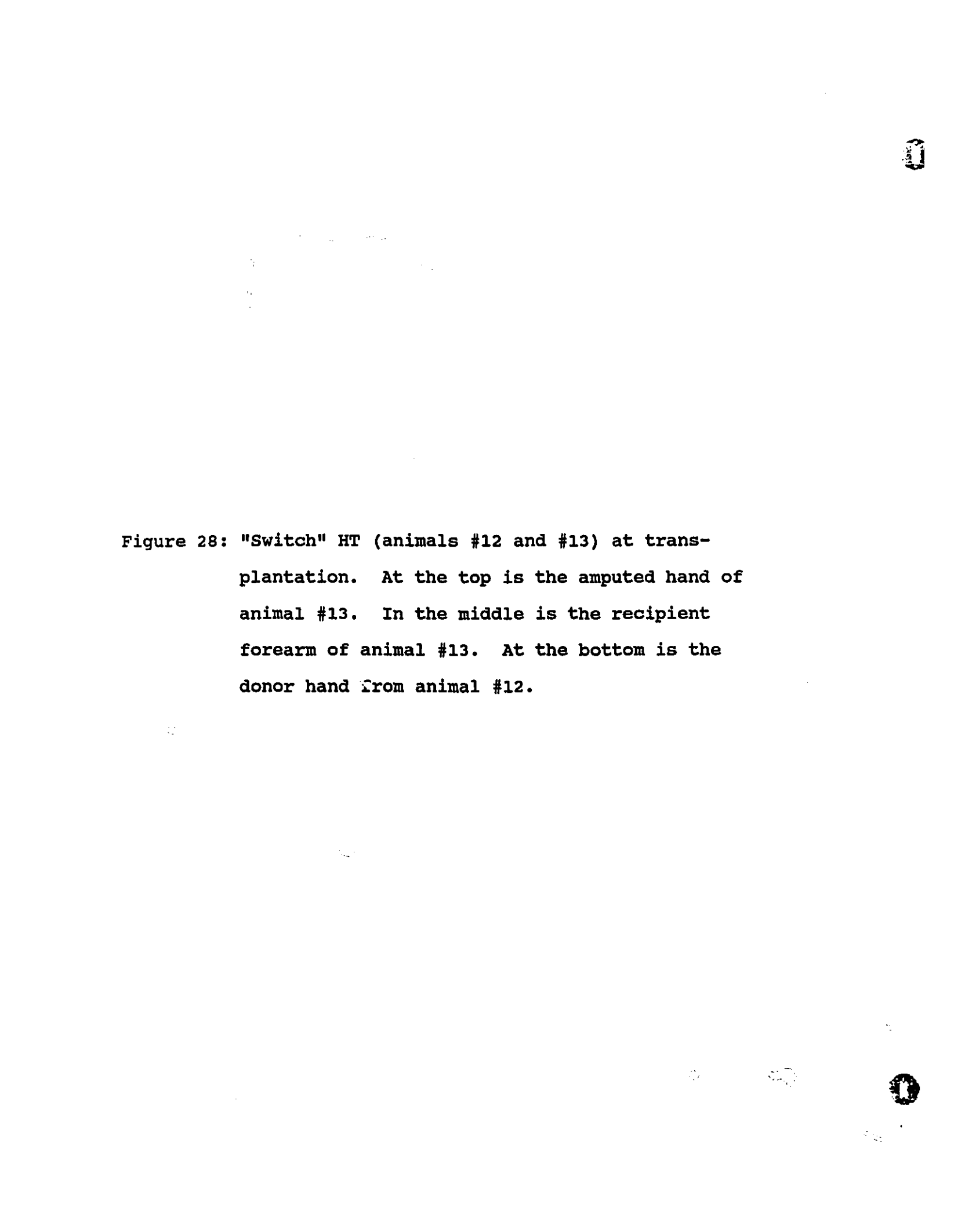


Figure 28: "Switch" HT (animals #12 and #13) at transplantation. At the top is the amputed hand of animal #13. In the middle is the recipient forearm of animal #13. At the bottom is the donor hand from animal #12.

Figure 29: "Switch" HT at completion of transplantation (animal #13).

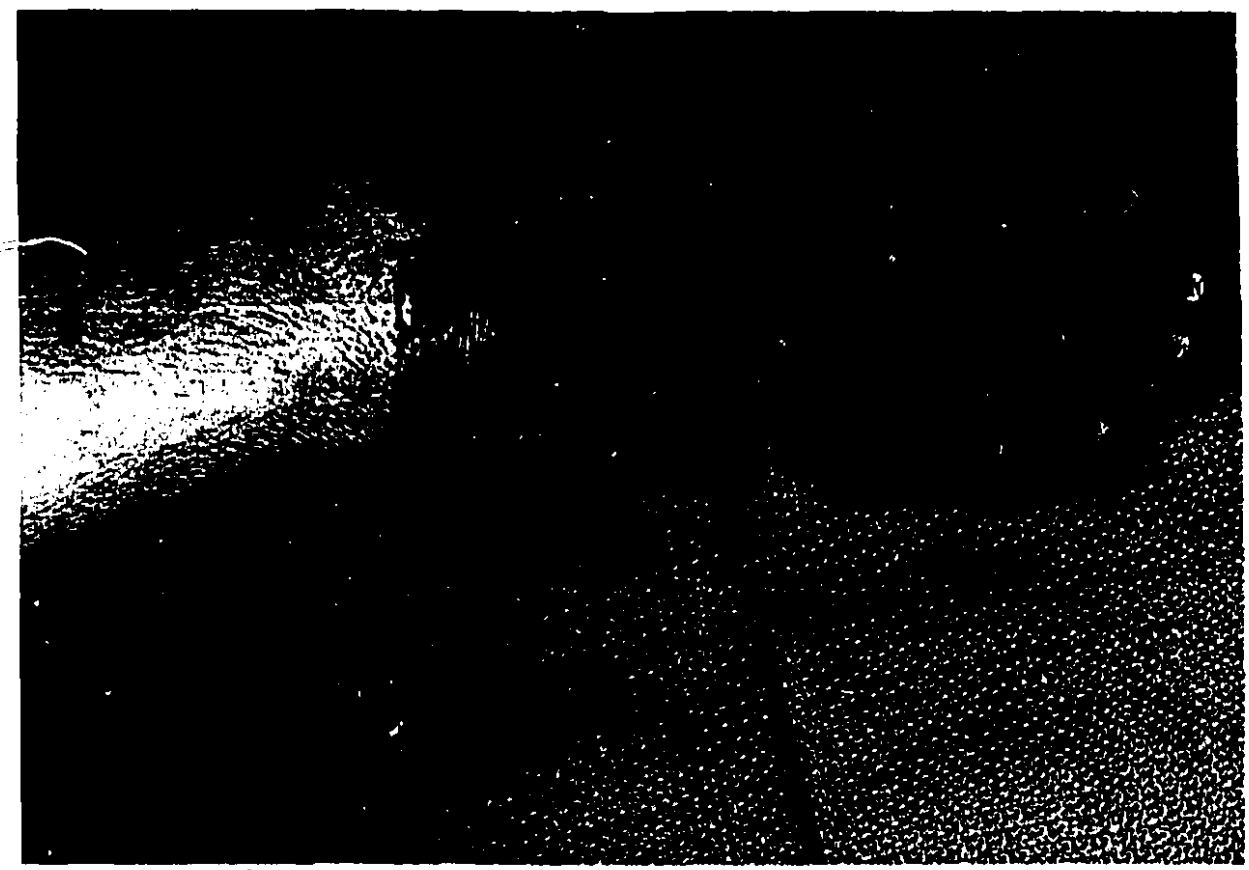


Figure 30: "Switch" HT at completion of transplantation (animal #12).

except for the one which underwent hyperacute rejection (#9). Although there was no record of skin ulceration at the injection sites, it is possible that these, if small, may have been missed for two reasons. First, baboons have very thick long fur easily obscuring lesions. Second, preoccupation with the difficulties in controlling rejection may have overridden attention to this detail so early on in our experience.

Steroids: We had experienced rejection problems in the first TNVFF (#5) and thus steroids were administered in the first two HTs (#7,#9) in response to obvious clinical signs of rejection rather than awaiting histologic confirmation. In the first HT (#7), although the CyA was increased on the day of the first dressing change (Day 5), the decision to implement the steroid rejection protocol was delayed to the following day for fear of infection. A skin biopsy was nevertheless taken at the next dressing change (Day 7), when visible improvement was already present, and repeated 13 days later (Day 20), when mild changes renewed concern. The results once again took several days to be reported, severely compromising their usefulness. In the first, there was evidence of some changes around blood vessels in viable skin, interpreted as moderate cellular rejection. In the second biopsy, the picture was similar, only improved, with evidence of fewer cells. In the second HT (#9), we did our first dressing change earlier (Day 4)

because of concerns at the time of surgery. At the end of the surgical procedure, we had noticed greater edema in the HT than seen previously and the beginning of a purplish discoloration along the transplant side of the incision line (Figure 31). Therefore, at the first postop verification, we became bolder by initiating steroid therapy immediately in response to worsening changes. A biopsy taken 7 days later was reported after a delay of a further week, as viable peeling skin without evidence of infiltration, a surprising result in view of the unrelenting clinically evident hyperacute rejection which took place in this animal despite massive doses of steroids (Figure 32).

In the remaining two HTs (#12,#13), which were chronologically done after the second TNVFF, (Tables 4 and 5) it became evident that steroids would have to become an integral component of the immunosuppression protocol, as used by some clinical transplant centers (13, 25, 29, 102, 127, 165, 215, 216, 217, 230). This was felt to be particularly true in this model where the antigenic load was of greater magnitude than in the TNVFF. Thus, steroids were started thereafter at the time of surgery.

1) Intraoperative results Intraoperatively the success of the microanastomoses was proven in all transplanted hands by the patency test (62). In addition, normal color, briskness, and quantity of bleeding were present in all cases from the proximal tissues of the

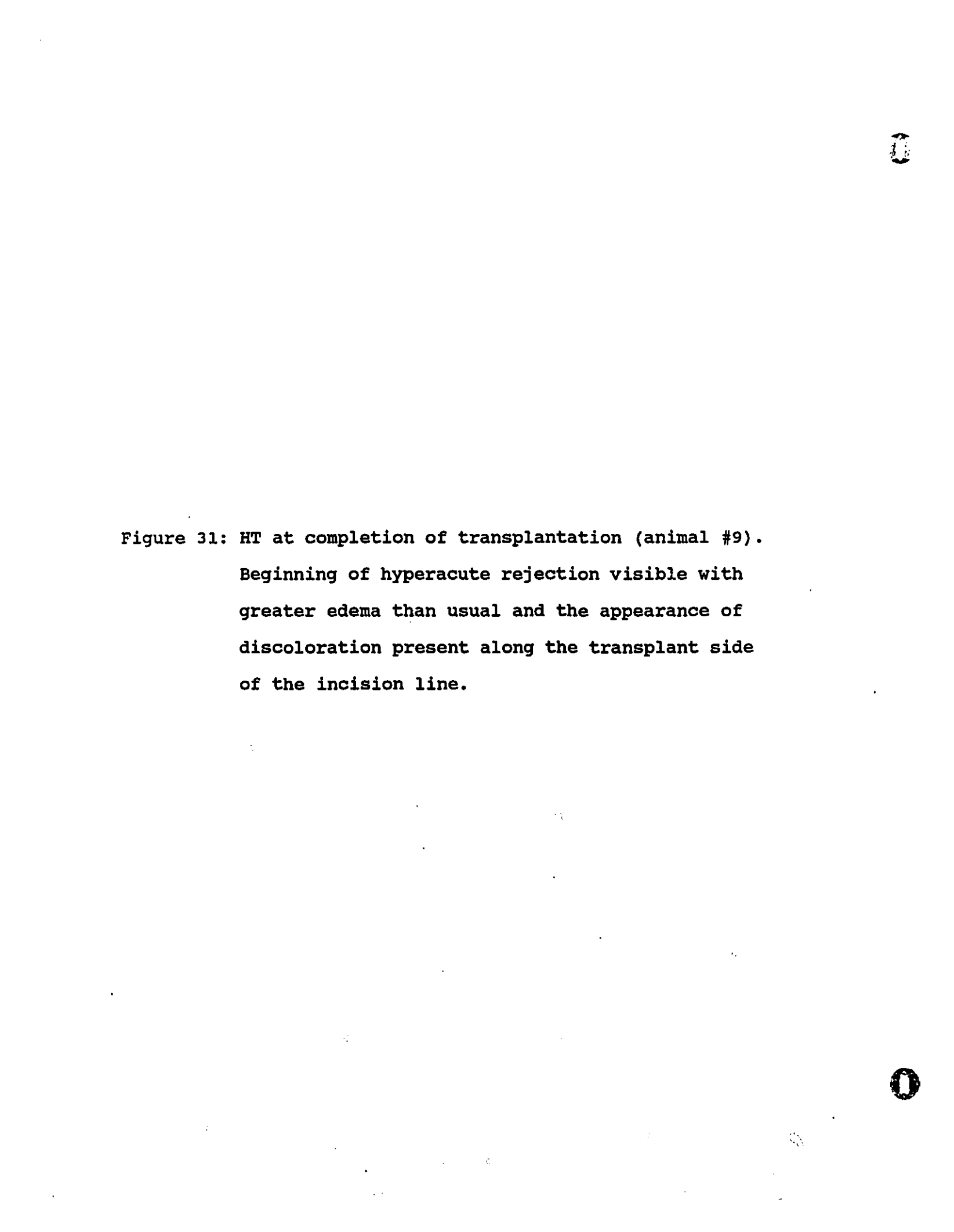


Figure 31: HT at completion of transplantation (animal #9). Beginning of hyperacute rejection visible with greater edema than usual and the appearance of discoloration present along the transplant side of the incision line.

Figure 32: HT 26 days post-transplantation (animal #9). Hyperacute rejection with necrosis and mummification of the fingers, and purulent material draining from the more proximal portions.


date: 30/5/84
day post-op: 26
animal: #9

transplant before hemostasis and from a stab wound to the volar epidermis of the hand at the end of surgery.

2) Postoperative results

A) Technical assessment Postoperative evaluation of each HT's viability was undertaken twice in the first week. In the case of the first transplant (#7), the first dressing change was arbitrarily done on Day 5. In the second (#9), it was done one day earlier because of concern with changes noted at the time of surgery. After the experience with hyperacute rejection in this animal, it was decided that all subsequent transplants would be checked on Day 3. This allowed two days for the animals to recover sufficiently from the long anesthetic to start eating before subjecting them to the side effects of tranquillization (drowsiness and anorexia) which could last the rest of the day.

All HTs showed evidence of good circulation based on assessment of color, turgor, temperature, and bleeding response to an epidermal stab wound. No transplant was lost due to technical failure of the microanastomoses.

B) Survival times Survival times for the HTs ranged from 26 to 311 days postoperatively (Table 5). Average survival time was 148.5 days. The two shortest survivals (#9,#12) were both being actively rejected at the time of euthanasia, although all or portions of each HT were still alive. In baboon #9, although hyperacute rejection had mummified all the fingers (Figure 32), the more proximal

portions, despite draining purulent material, still showed the presence of blood circulation. In HT #12, all portions were still viable at the last dressing change despite weeping, infected surfaces (Figure 33). Only two transplants (#7,#13) survived long enough in a relatively stable state with persistent mild to moderate rejection to warrant assessment of reinnervation by our colleagues using neurophysiologic recording techniques (58, 81, 199).

Undertaking these highly immunogenic transplants later on in this project, when greater experience had been gained, might have provided longer survival times. Although the number of HTs is low, it seems that their successful transplantation may be significantly more difficult to achieve than that of TNVFFs because of rejection.

C) Rejection None of the four HT passed the first few days without clinical signs of rejection (Table 5). Signs of mild, moderate, and severe rejection were the same as for the TNVFF with one addition. In the early moderate cases of rejection, seen in two animals (#12,#13), bullae appeared (Figure 34) which went on to become localized areas of breakdown. In the one case of hyperacute rejection, as mentioned above, changes began at the conclusion of surgery. The hand was more edematous than usual and a purplish, erythematous discoloration was present along the proximal edge of the transplant (Figure 31). Its unrelenting course included increased swelling and a brownish erythematous

Figure 33: HT 69 days post-transplantation (animal #12).
Severe rejection with weeping, infected surfaces.

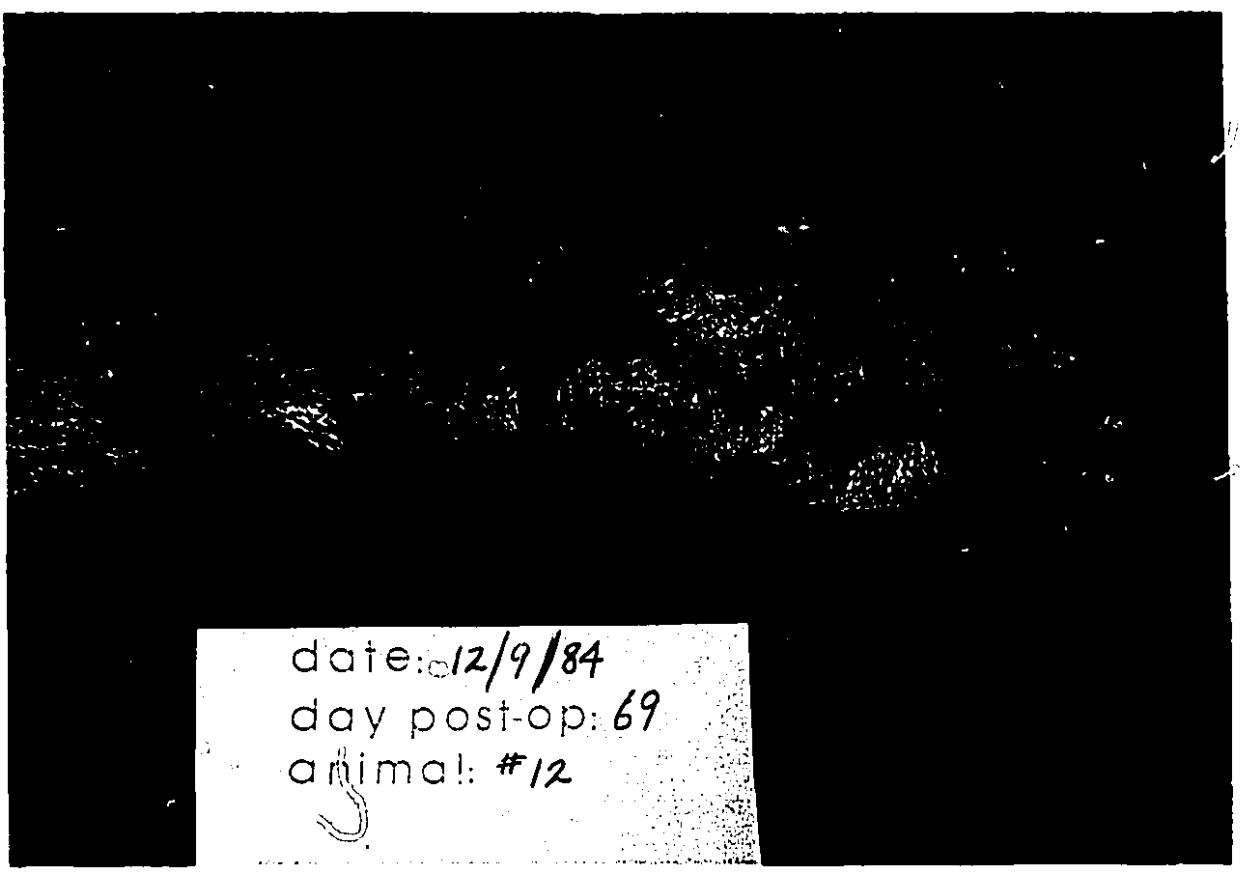
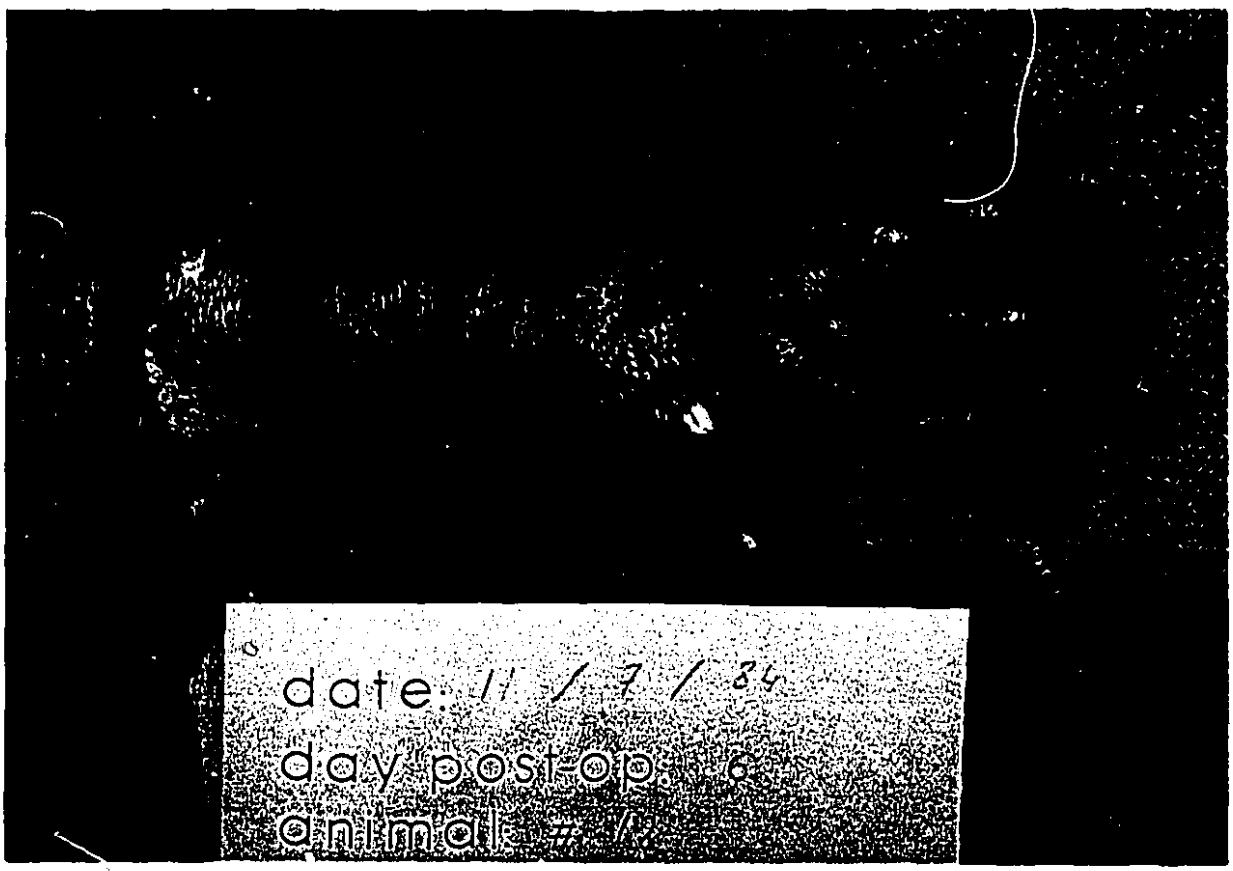

date: 12/9/84
day post-op: 69
animal: #12

Figure 34: HT 6 days post-transplantation (animal #12). Early moderate rejection with appearance of bullae.


date: 11/7/84
day post-op: 6
animal: #12

discoloration with multiple small vesicles in the proximal portion of the HT, followed by the appearance of a few bullae. Sudden massive desquamation of the palm and fingers occured next, quickly ending with necrosis and mummification of the fingers (Figure 32). This series of changes happened despite restarting high dose steroids on Day 11 after having first initiated it on Day 4. What seemed at the time to be adequate doses of CyA (starting with 22.1 mg/kg/dose twice daily), in retrospect proved to be insufficient and it is clear that our worries about toxicity dominated our readjustments at a time when we were not yet familiar with the pharmacokinetics of the drug. We never attained a level greater than 840 ng/mL and even dropped as low as 390 ng/mL subsequently. Despite these factors, and suggested by the very early onset of rejection, it is possible that this transplant would not have been salvageable even if it had been undertaken after greater experience with more aggressive immunosuppression.

Nevertheless, in most of the HTs the rejection protocol did prove to be useful in controlling and even temporarily reversing the rejection process. Although this model appears to possess a much greater immunogenicity, resulting in greater difficulty in preventing rejection, refined use of CyA and steroids have not been tested thoroughly enough in this experiment to determine whether or not results could be further improved.

3) Summary Since 2/4 transplants (#7,#13) (Figures 35 and 36) survived for a prolonged period of time despite several rejection episodes and lack of experience, further sophistication with this model or the addition of more potent immunosuppression may still translate into greater success.

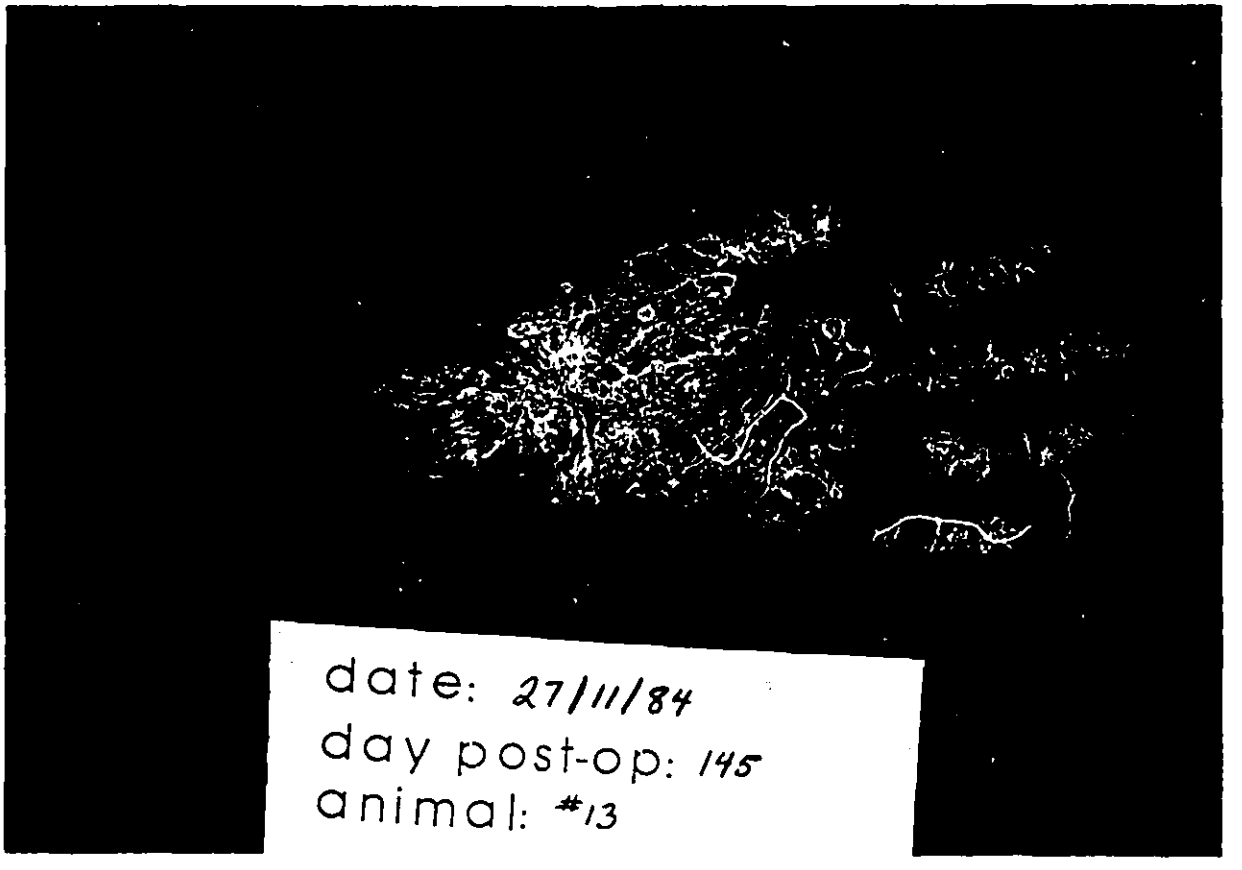
As in experiment #7, having to transfer the daily care of the animals, using the Cremaphor-EL vehicle for subcutaneous CyA administration and encountering difficulties with the CyA RIA, may have all had some effects on the results. It is nevertheless the impression of the author that this model is intrinsically more difficult to use because of the magnitude of the immunologic load.

During the course of this experiment, several significant points became evident and were put to use in the latter part of Experiment #7. These included the importance of the preoperative administration of CyA in order to achieve adequate blood levels at the time of transplantation. For HTs perhaps one week of twice daily injections at a dosage of 23 mg/kg/dose would be appropriate or perhaps a longer period with a lower dose would be more successful. The need for steroids as an essential part of the immunosuppressive regime, as used by some clinical centers (13, 25, 29, 102, 127, 165, 215, 216, 217, 230), was clearly established. Perhaps they too should be started preoperatively and tapered more slowly. Although

Figure 35: HT 308 days post-transplantation (animal #7). Mild to moderate rejection with build up of a thick, firm, epidermal layer.


date: 25/01/85
day post-op: 308
animal: #7

Figure 36: HT 145 days post-transplantation (animal #13). Moderate chronic rejection with persistent flaking.


date: 27/11/84
day post-op: 145
animal: #13

appropriate CyA serum trough levels seem to fall within the same range as for the TNVFFs, the author would prefer keeping them in the 1000-1500 ng/mL range (aiming for just over 1,000 ng/mL) using the room temperature assay technique. Prevention of all rejection episodes should be the goal and immediate aggressive response to even the slightest changes should be the rule.

Two HTs (#7,#13) survived to beyond the 4 months it normally takes for reinnervation of the distal tip of the fifth digit following transection of the ulnar nerve at the wrist (229). The model was therefore successful in fulfilling the goals of the project despite the difficulties encountered.

DISCUSSION

The aim of this project was to design two models in the primate for reconstructive surgery utilizing vascularized free tissue transplants with nerve repair. In so doing the answers to two questions were sought. First, can limbs or portions thereof be transplanted successfully between entirely unrelated individuals of a primate species? Second, can the newly designed primate transplant models successfully achieve sufficiently long survival for functional motor and sensory reinnervation to have occurred? Additionally, is reinnervation of the transplanted tissue by recipient nerves under CyA immunosuppression possible? In order to evaluate this study in light of these parameters each point is best addressed separately.

From a purely design point of view, the two models were entirely successful. No transplant or replant was lost due to a technical or anatomic problem. During the course of the experiments, modifications helped refine surgical details. The resultant final models were relatively simple, very dependable and easily reproducible.

The choice of animal species for this study, although associated with some inconveniences, did allow us to achieve our goals. Anatomic differences from humans, for the purposes of these experiments, did not complicate design. Neurovascular and musculoskeletal structure size present in

the female Papio hamadryas anubis was appropriate for the needs of the project.

One possible modification would be the use of male baboons. This may avoid potential hematologous infection of the transplant from lesions on the buttocks when females are estrous. Most importantly, the possibility of pre-sensitization of a recipient by the MHC through pregnancy would be eliminated (which may have been a factor in baboon #9's rejection of the hand transplant since previous pregnancy is known to be a causative factor in hyperacute rejection (130)). The effects on animal care (e.g., sizes of cages and quarters, difficulty in handling) and size of anatomic structures would have to be evaluated.

Although not all the animals underwent histo-compatibility testing, the prevalence of rejection suggests that the transplants were undertaken between non-identical pairs. Of the six animals that were tested in order to choose four donor-recipient pairs (#16-#17, #16-#20, #18-#19, and #18-#21), i.e., two animals donated two TNVFF each to the remaining four, three pairs were identified as unquestionably different and the remaining pair had a high probability of being different. These pairs were purposely picked on the basis of incompatibility to avoid the potential criticism that transplant success resulted from accidental histocompatibility matching.

The experiments did prove that limbs or portions thereof can be transplanted successfully between unrelated individuals of a primate species. One term however, does need to be qualified, that being success. If measured purely in terms of transplant survival times, the results were impressive considering the average survival of 179 days (ranging from 26 to 413 days). These numbers are all the more significant since the end point for each transplant was never considered to be loss of the whole allograft from rejection. In most cases, termination of the experiment was determined using electrophysiologic evaluation by our neurophysiologic colleagues or euthanasia for illness. Thus, despite the presence of chronic rejection in many cases, the transplants could probably have survived for indeterminate additional periods of time.

The survival times are difficult to compare to those in the rat limb transplant experiments (10, 11, 92, 93, 115, 129). Only one group administered CyA to some rats continuously (minimum 44 days, maximum 113 days) (92, 93). There is little information on the outcomes of this group, although the authors did imply that the cardiac puncture used to sample blood for the antibody titers significantly affected survival times. The only other information available on these animals is that five out of seven animals showed no clinical, immunologic or histologic evidence of

rejection and that the remaining two had respectively mild and moderate histologic changes.

In another article, a single rat is described with a transplanted limb surviving for a seemingly indefinite period of time (no visible signs of rejection at publication) to Day 225 despite ending CyA administration on Day 20 (115). This example supports the concern expressed under Objectives and Research Plan about the significance of transplantation experiments between inbred strains of rats and their extrapolation to higher species. Such easily induced tolerance may also explain the apparent lack of any clinical signs of rejection during relatively low dose CyA treatment and even beyond. In contrast, in this primate study, the survival times were not without ongoing significant battles against rejection and side effects. This brings up the less positive side of the "successful transplantation" issue. Long enough survival times in most animals for reinnervation to have taken place (assuming it occurs and progresses at a speed similar to that of nerve regeneration following injury in the absence of immunosuppressants) was achieved. But, at what expense?

Despite CyA dosages with resultant serum trough levels of at least four times that recommended for clinical organ transplantation (28, 37, 127, 230, 233) by Sandoz (Canada)

and Dr. Shumway's laboratory,[23] and the additional use of steroids, rejection had been a constant concern and threat. All the HTs and 4/7 of the TNVFFs had undergone significant episodes of rejection. Of course, these numbers must be taken in light of several factors. First, no attempt at histocompatibility matching was made deliberately. Although the effects of matching in this type of transplantation is unknown, it would be worthwhile to undertake similar transplants between animals of known unrelated lineage (such as those of experimental baboon colonies) that have been matched for histocompatibility, as is done in human kidney transplantation. Second, the HTs, which are probably more difficult to handle because of a greater antigenic load and the variety of tissues present, were undertaken very early in our learning curve. The results for these may have been better had they been attempted later in the series when our knowledge and experience were greater. Third, in a more general sense, it is possible that repeating the whole study with our present level of experience would achieve a more stable postoperative rejection-free course with the same immunosuppressive regime, or better yet, using newer more sophisticated methods (104, 127, 177, 191) or newer drugs.

Although all these factors probably played a part in limiting our results, two issues should not be overlooked.

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[23] Dr. Norman E. Shumway's Laboratory [verbal communication], Stanford University Medical Center, Stanford, California, U.S.A. 94305

When setting out to develop these models, it was not even clear if any degree of survival would be possible. Clearly this point has unquestionably been resolved. It is possible that additional immunosuppressive techniques might not only be useful in such transplants, but also necessary. It is also conceivable that the "right" immunosuppressant has yet to be developed in order to facilitate this type of transplantation.

The need to search for other better methods of immunosuppression for the transplantation of primate limb composite tissues is further underlined by the excessively high incidence of side effects in this project. Although beyond the scope of this thesis to undertake a detailed analysis of all the side effects and attempt to explain their possible etiology, their mention is necessary in the overall assessment of the models. They included: anorexia, anemia, gingival hyperplasia, hepatotoxicity, hirsutism, lymphoma, nephrotoxicity, subcutaneous and intramuscular abscess formation, and tremors.

Some side effects were noted in every animal at one time or another such as anorexia (52, 87, 194, 214, 232) and weight loss (52, 165, 194, 232). Although these symptoms seemed related to CyA administration, it was impossible to isolate this as the cause since rejection and almost any other illness could share this sign.

Other problems such as anemia (37, 87, 194), although seen in several animals to some degree, became catastrophic in few. Only one animal (#19) passed away from an unrelenting drop in hemoglobin to 15 g/L.

Gingival hyperplasia was more than a nuisance (24, 26, 28, 29, 37, 52, 87, 102, 165, 194, 216, 230, 232). Nearly half of all animals (all but one with transplants surviving over 150 days) developed this problem. Although judicious tooth brushing was instituted at each dressing change as soon as signs of inflammation appeared (not earlier for fear of causing irritation and stimulating the process), this method proved to be ineffectual. Two animals developed such florid hyperplasia that gingivectomy had to be undertaken while others would probably have benefitted from this procedure.

Hepatotoxicity (24, 26, 28, 29, 37, 73, 87, 89, 102, 106, 110, 165, 194, 204, 214, 216, 230, 231, 232, 233) was difficult to identify since elevated liver enzymes were noted in most of the animals pre-transplantation and seemed to fluctuate afterwards in ways difficult to interpret.

Hirsutism (24, 26, 28, 29, 37, 87, 102, 165, 175, 194, 214, 230, 232, 233), although noticeable in some of the animals, was impossible to quantify in these primates with normally thick fur coats.

Of particular concern was the finding of a histologically proven lymphoma (24, 26, 28, 29, 37, 165, 175,

177, 191, 194, 216, 230, 232, 233) in one animal (#17) diagnosed clinically by the appearance of enlarged lymph nodes in the lower limbs and an enlarged spleen.

Nephrotoxicity was not as common as expected (8, 24, 26, 28, 29, 30, 37, 87, 102, 106, 110, 121, 127, 131, 165, 175, 177, 190, 194, 195, 204, 214, 215, 216, 217, 230, 231, 232, 233). Only one animal developed irreversible renal failure (#5) and only one other (#11) showed a transient slight increase in creatinine to above 142 μmol/L, considered to be the upper limit of the normal range for baboons (223).

Although three (#11,#19,#21) animals developed spontaneous subcutaneous abscesses which generally grew staphylococcus aureus, a much greater problem was the development of sterile intramuscular abscesses in all subjects (3). These developed in response to repeated injections of CyA dissolved in its lipid vehicle and led to intramuscular fibrosis, seen histologically (52) and noted also in Dr. N.E. Shumway's laboratory. It is believed that this phenomenon played a significant part in preventing long-term stable rejection-free transplant status in those animals that did not develop life threatening side effects. Although difficult to prove, since too many variables fluctuated during the course of the study (methods of administration of CyA, levels of CyA aimed for, difficulties with the RIA, and modifications in the technique of the

RIA), it is the author's impression that, with time, controlling CyA serum levels became increasingly more difficult, resulting in swings in trough levels. This could be explained by the development of ever increasing zones of fibrosis in the limited muscle mass present, from which absorption of the drug would be erratic. In turn this would result in further increases in dosage (and volume) thus compounding the problem.

Although transient tremors were noted in some animals, they were not reliably recorded in the charts since they were often discounted as being chills related to fever. It is therefore not possible to comment on neuroesthesias, known to occur in human CyA treated patients (24, 26, 28, 29, 37, 87, 102, 165, 175, 190, 214, 230, 232, 233).

This brings the author to reflect on a few points with respect to the use of animals in laboratory research in general and more specifically, in projects such as this one in which there are potential side effects and some degree of unpleasant manipulation. Very attentive care, around the clock when necessary, by a few dedicated caretakers not only reduces animal stress but insures consistency of care. Careful observation and handling probably contributed to the success of this study since we did not encounter any of the aberrant patterns of behavior (e.g., psychosis, automutilation) observed by some scientists working with

this species including Dr. Robert Dykes,[24] head of the neurophysiology team that studied the transplants and has previously worked with this species (79, 229). Recipients were all carefully chosen favoring agressive or indignant behavior. Animals demonstrating cowardly responses, uncontrollable nervousness or inappropriate conduct were assigned as donors. It was felt that a demeanor suggestive of a strong character would best be suited to the experimental conditions. In addition, attention was given to details of animal comfort. This included ensuring that all unpleasant manipulations were rewarded, that adequate analgesia was always available and that the animal quarters were kept as interesting (e.g., television, radio, toys), pleasant, and clean as possible. The animals were handled gently but firmly to avoid injury, noting any changes in their physical signs and comportment. These details may seem trivial, but probably played an important role in the outcome.

It is therefore not surprising that these two models had the severe drawback of being very expensive. Not only were they costly monetarily (i.e., animals, cages, quarters, care, surgical equipment, dressings, medications, etc.), but they were time-consuming, labor-intensive, and emotionally

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taxing because of the multitude of details to balance and the conscious focus on animal welfare.

These two models, despite their inherent difficulties, were developed to provide the means to answer the question: Can functional motor and sensory reinnervation of foreign tissue by recipient nerves in the primate occur in the presence of immunosuppression (specifically CyA and steroids)? Although the detailed answer to this question is beyond the scope of this thesis, our neurophysiologist colleagues have used these models and found the answer to be YES (58, 81, 199).

Two HT and 5 TNVFF have been analysed by them. In simple terms, not only was sensory reinnervation shown to occur in a manner similar to that seen following nerve injury, but intrinsic muscle contraction was possible in response to motor stimulation and even joint proprioceptor afferents were identified. Not surprisingly, the quality of reinnervation was greatly dependent on the extent of rejection having taken place.

Thus, not only have these models successfully achieved the goals set out on paper in the beginning, but they have already been put to use by colleagues in answering the most basic question in reconstruction by transplantation, that of reinnervation.

CONCLUSION

A further step has been taken on the road towards clinical reconstruction by transplantation. Two composite tissue models, one for the study of sensory reinnervation with a smaller variety of antigenic tissues, the other for the study of mixed motor and sensory reinnervation comprised of a larger, more varied antigenic load, have been developed in the primate. Although long term survival (beyond 4 months) was achieved in nine of 11 animals, it has not been without considerable effort in controlling and preventing rejection and battling significant side effects. There is no question that immunosuppression requires further perfection before these models can be reproduced with little or no effort. Now that these models have been analysed by our neurophysiological colleagues and shown to undergo reinnervation, further laboratory work in perfecting these models takes on greater pertinence. This next step of course, is intimately related to developments in the field of immunosuppression, confirming once again the interrelationship of transplantation and reconstructive surgery.

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APPENDIX I

The Veterinary Health Certificate accompanying each animal certified that the animal in question was "free from visible symptoms of infections, contagious or communicable disease" and stated the date of the last negative tuberculin test. Acute animals were retested for tuberculosis once. Despite a negative result, they were treated and disposed of as if contagious. Long-term individuals were quarantined for a period of four weeks. During this time period, they were subjected to a series of three tuberculin tests at two week intervals, to confirm negative status.

ROUTINE PRIMATE PROCEDURES*

- All imported Rhesus and Cynomologus monkeys,** upon arrival, are given the following routine procedures:

T.B. Test:
An intra-dermalpalpebral injection of Koch's old tuberculin is given in the upper eyelid. The dose is 1/10 ml per monkey. Observations are made at 24 hours, 48 hours and 72 hours to pick up the reactors. Tuberculin testing is carried out every two weeks until three consecutive negative tests are obtained. Thereafter, T.B. testing is done at four weeks interval.

Immune Serum Gamma Globulin:
One ml of immune serum gamma globulin (Human) is administered intramuscularly in one leg.

Long Acting Penicillin:
One ml of 300,000 units of long acting penicillin is injected intramuscularly in the other leg.

Deworming:
Two ml of Equizole A (preparation containing Thiabendazole and Piperazine) or Camvet (containing Cambendazole), both products of Merck Sharp and Dohme, is given orally.

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*Extract from "Routine Primate Procedures", Charles River Research Primates Corporation, P.O. Box 416, Port Washington, New York, 11050, U.S.A.

**According to Michael A. Nolan, President of Charles River Research Primates Corporation, this protocol is also used for baboons.

Electrolytes:
A pinch of electrolyte powder is added to each monkey's water bowl daily, for three days, to restore ionic balance which might have been upset due to stress and strain of transportation.

- During the period of quarantine, diseases and minor ailments are treated according to the condition. However, a few therapies on frequently encountered conditions are given below:

a) Off Feed: (Not eating) This is generally due to respiratory problems or a result of stress and strain of transportation. One ml of 300,000 units penicillin intramuscularly daily, for three days, helps in most of the cases. If the animal is prostrate, one ml of corticosteroids, such as Prednisolone or Cortisone, along with appropriate antibiotics, should be given. The diet is supplemented with sustagen - a preparation containing proteins, carbohydrates, fat, minerals and vitamins. Juvenile monkeys are often put on this feed supplement.

b) Respiratory Problems: (Including Pneumonia) Never one single drug has been found effective. According to symptomology, the following broad spectrum antibiotics are used:
1) Penicillin
2) Achromycin
3) Ampicillin
4) Cephalothin
5) Chloramphenicol.

Prolonged treatment with antibiotics is avoided for obvious fear of monillial problems. Corticosteroids may also be given along with antibiotics.

c) Enteric Problems: Furoxone suspension containing Furazolidone in doses of 5-10 cc (according to the weight of the monkey), orally, has been found efficacious in taking care of most of the enteric problems. Obstinate cases, like respiratory problems, should be evaluated on symptoms manifested and antibiotics, chemotherapy should be used out of the undermentioned items:
1) Entromycin
2) Garamycin
3) Achromycin
4) Septra/Bactrim
5) Kanamycin
6) Metronidazole (specific for cases of Amoebiasis)
7) Diodohydroxyquin (specific for cases of Amoebiasis).

d) Intestinal Parasites:

1) Equizone A/Camvet are efficacious in cases of ascaris, strongyloides, hookworms and other nematodes.
2) Phthalofyne (Whipicide) is a good product for whip worms - Trichuris trichuris.
3) Pyrvinium Pamoate (Povan) is a good product for pinworms.
4) Niclosamide (Yomesam) or Bunamidine Hydrochloride (Scolaban) may be used in cases affected by tapeworms.

e) Eye Infections: Terramycin/Chloromycetin ophthalmic ointment should be used.

f) Tranquilization/Restraint: Ketamine Hydrochloride or Phencyclidine Hydrochloride (Sernylan) are effective drugs for restraint. We prefer Sernylan since it brings about a longer period of tranquility/anesthesia by using small dosage. These drugs are very useful for immobilizing monkeys to carry out diagnostic examination, minor surgical procedures and restraint of large animals.

- A copy of each of the undermentioned product information is enclosed for future reference to work out dosage and study indications:
Immune Serum Gamma Globulin; Long Acting Penicillin; Equizole A; Camvet; Electrolyte; Sustagen; Prednisolone/Cortisone; Achromycin; Ampicillin; Cephalothin Chloramphenicol; Furoxone; Entromycin; Septra/Bactrim; Garamycin; Kanamycin; Metronizadole; Diodohydroxyquin; Whipicide; Povan; Yomesam; Scolaban; Ketamine and Sernylan.

APPENDIX II

Experimental Models in Primates for Reconstructive Surgery Utilizing Tissue Transplants

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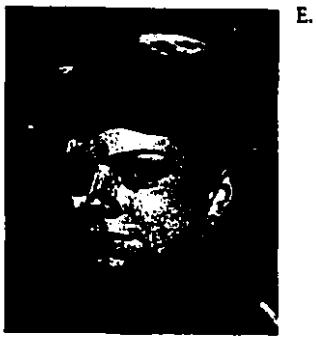

E. Patricia Egerszegi

Experimental Models in Primates for Reconstructive Surgery Utilizing Tissue Transplants

E. Patricia Egerszegi, M.D.,
Donald D. Samulack, B.Sc.,
and Rollin K. Daniel, M.D.

Two experimental models for tissue transplantation between unrelated individuals of a primate species have been designed to study survival and reinnervation. The first is a neurovascular free flap consisting of the entire soft tissue coverage of the index finger. The second is an entire hand transplant through the distal forearm. Ongoing studies show that cyclosporin A at high doses, in combination with a tapering regimen of steroids to a low maintenance level, permits prolonged survival of both transplant models. Careful biochemical, hematological, and cyclosporin A serum trough level monitoring permits use of this drug at very high dosages in primates. Continuing experiments should yield detailed neurophysiological data on the reinnervation of these transplants over the next 6 to 18 months.

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The primary goal of reconstructive surgery is restoration of function and quality of life to patients with congenital or acquired deformities. Numerous operative procedures have been developed utilizing skin grafts and skin flaps, as well as grafts of bone, muscle, and nerve. However, problems remain for those patients in whom there is insufficient or inadequate donor tissue to reconstruct the missing structure. For instance, young adults who suffer nonreplantable upper extremity amputations frequently discard functional prostheses. Children who sustain severe facial burns can be covered with skin grafts, but the resulting deformity severely affects their quality of life. As these problems cannot be corrected with the patient's own tissue, the only solution is transplantation of comparable tissue from donors.

Recently a new drug, cyclosporin A (CyA), has permitted dramatic breakthroughs for immunosuppression following tissue transplantation. The drug has been found effective clinically in kidney [4, 5], heart [18], lung [19], liver [4], pancreas [20], and bone marrow [13] transplantation. Experimentally, it has allowed transplantation of other organs and tissues, including nerve [27, 28], muscle [11, 25], bone [23], and skin [3, 10, 16, 26]. The superiority of CyA over other immunosuppressive regimens makes the successful transplantation of tissues for reconstructive purposes a realistic goal. However, these transplants differ significantly from previous clinical transplants in that sensory reinnervation is essential. Unlike renal and heart transplants, which maintain their activity independently of direct neural regulation, digits and hands must obtain functional reinnervation to be useful. We have therefore undertaken the design of two experimental models in the primate to study the survival and reinnervation of transplanted tissues.

Review of Literature

Throughout its evolution, reconstructive surgery has had a close association with the transplantation of tissue. In 1863, Bert [18a] studied skin allografts and xenografts as well as autografts and reportedly noticed different degrees of success for each. These empirical observations became the basis for transplantation medicine and the first of an increasing number of studies. In the 1950s, Converse was instrumental in initiating the first international meeting on transplantation and, with his collaborators, in documenting normal human skin allograft behavior [5a]. Their work also clarified some basic principles of immunology, which eventually led to advances in understand-

ing the importance of histocompatibility [19]. The successful transplantation of a kidney between identical twins in 1954 by Murray et al [17], a plastic surgeon at the Peter Bent Brigham Hospital, demonstrated the feasibility of transplantation; its wider application awaited only the development of a form of immune modulation that would reliably prevent rejection.

In the 1960s, major steps were taken with the introduction of 6-mercaptopurine and then azathioprine. Prednisone was soon added to the latter to achieve the additive effect of two drugs with different modes of action. In 1969, 2 new strains of fungi were discovered in the Microbiology Department of Sandoz (Basel, Switzerland) Ltd. that produced a metabolite showing evidence of potent immunosuppressant potential with very low toxicity. Following the first publications documenting this drug's effectiveness in transplantation, cyclosporin A, as it became known, has been utilized increasingly as an experimental immunosuppressant for the transplantation of numerous organs and tissues. Most significantly, CyA has permitted successful long-term skin allograft survival in various animal models [3, 10, 16, 26].

Skin, as the major barrier between individual and environment, has been found not only to be more immunogenic than the kidney, for example [21], but also to undergo rejection more readily than the other tissues present in a limb [15]. This finding may explain the greater CyA requirement for skin allograft survival as compared with that for certain solid organs [1, 26]. Skin flaps have never been studied utilizing CyA immunosuppression and studied very little using other modes of immune modulation [22]. Three groups have addressed the question indirectly by transplanting entire rat limbs, with their skin cover, using CyA [2, 7, 8, 12, 14].

In 1982, Black et al [2] published the first data on survival of adult rat limb transplants with CyA immunosuppression, followed a year later by their final results [12]. Five groups of rats were studied. The first 2 served as controls, undergoing transplantation without treatment or receiving the solvent alone. In the next 2 groups, a donor-specific blood transfusion was administered 1 week prior to transplantation, with the addition of passive enhancement on days 0, 2, and 3 in the fourth group. The last group was treated with 25 mg of CyA per kilogram of body weight per day subcutaneously for 20 days. Despite being known to prolong kidney allograft survival across even stronger histocompatibility barriers, the donor-specific blood transfusion did not significantly prolong limb allograft survival even if combined with passive enhancement. Yet there was a prolongation of graft survival in at least one animal with CyA to greater than 225 days, a dramatic result when compared with either control group.

Fritz et al [7, 8] also achieved prolonged survival following heterotopic transplantation of adult rat limbs across a strong antigenic mismatch. All groups, except controls, received 10 mg of CyA per kilogram of body weight per day subcutaneously. One group received the immunosuppressant for 7 days, another for 21 days, and the last continuously. Controls underwent clinically evident rejection within 14 to 20 days, whereas only 2 out of 5 animals in the short-term treatment group showed similar signs 4 to 5 weeks after discontinuation of therapy. None of the treated animals showed any strong histological evidence of rejection, and a total of 9 treated rats had no evidence of rejection at all. This finding was in contrast to that for untreated rats, all of whom had strong histological evidence of rejection. The results from antibody testing correlated with the histological findings. All the untreated animals had a strongly positive antibody response, in contrast to none of the continuously treated animals.

Kim et al [14] recently published a similar study in which adult rat limbs were transplanted orthotopically across defined histocompatibility barriers with varying regimens of treatment. Of 6 groups, the first underwent limb replantation and the second transplantation, both without immunosuppression. The next 2 groups received 10 mg per kilogram of body weight per day of intramuscular CyA, one for a period of 2 weeks and the other for 2 months. The remaining two groups received 10 mg per kilogram of body weight per day of intraperitoneal azathioprine and prednisolone, respectively. The replanted limbs showed some edema during the first week and denervation atrophy, but otherwise had a normal long-term appearance. In the control group, clinical signs of rejection appeared on the average at 6 days. The short-term CyA group showed some edema during the first 7 to 10 days, followed by normal appearance until 16 to 22 days beyond the end of treatment. In the long-term CyA group, appearance of the limbs was similar to the replants until 6 to 10 days beyond the end of treatment, when signs of rejection appeared. This result was in contrast to the azathioprine and prednisolone groups, in which rejection appeared on the average at 7.2 and 7.8 days, respectively, during continuous therapy.

In summary, CyA was shown to prolong sig-

nificantly the survival of whole limb transplants in the adult rat. Nevertheless, the question remains as to whether transplantation between inbred strains of rats poses sufficient challenge for allograft survival and whether findings in this lower species can be extended to human patients. The rat limb transplantation studies, nevertheless, have provided an impetus to answer these critical questions: (1) Can transplanted tissue undergo *functional* motor and sensory reinnervation with CyA immunosuppression, and (2) can limb transplantation be successful in entirely unrelated individuals of a primate species?

Our laboratory has begun an investigation into the degree of reinnervation and functional recovery possible following transplantation with CyA in a primate species. The baboon, specifically *Papio anubis*, was chosen as the experimental animal for the following reasons: (1) The hand is anatomically similar to that of man; (2) the skin contains similar sensory receptors and nerve pathways to those of man; (3) the neurovascular bundles are of sufficient size to allow microsurgical repair and neurophysiological recordings; (4) the baboon hand has been used previously in our laboratory for assessing neural function, thus allowing utilization of established techniques and protocols [24]; (5) CyA has been used successfully in several primate species in organ transplantation; and (6) there exists a reliable source for these animals.

Two models have been designed as the ultimate challenge in allograft survival: a neurovascular free flap consisting of the entire soft tissue coverage of the index finger and an entire hand transplant through the distal forearm. These designs provide the opportunity to evaluate not only skin, soft tissue, muscle, and bone survival but also, most importantly, nerve regeneration and ultimate function. The goal is to assess whether sensory and motor nerves are able to reach the appropriate receptors in foreign tissue and establish functional contact.

Materials and Methods

Perioperative Management

Each animal received prophylactic procaine and benzyl penicillin (11,000 IU per kilogram of body weight per day of each), streptomycin (27.5 mg per kilogram of body weight per day), and netilmicin (50 mg twice daily), all intramuscularly. Sterile surgical technique was employed. Following tranquilization with ketamine and xylazine, anesthesia was maintained with intravenous sodium pentobarbital. Urinary output and heart rate were monitored to maintain hemodynamic stability. Ventilation via an endotracheal tube was self controlled using 100% oxygen adjusted to provide minute ventilation. Expired carbon dioxide concentration and rectal temperature were maintained within physiological range. Prior to beginning the microvascular anastomoses, each animal received a single intravenous dose of heparin (70 IU per kilogram of body weight). Following dressings, a rigid thermoplastic upper limb splint was utilized for protection. Postoperatively, acetylsalicylic acid at a dosage of 75 to 100 mg orally was given daily for 10 days. CyA was administered by intramuscular injections, beginning with 14 mg per kilogram of body weight twice daily (dissolved in Miglyol 812 and absolute ethanol) 3 days prior to surgery. The drug treatment was continued for the length of each study with dosages adjusted to maintain minimum serum levels around 800 ng per milliliter (as monitored by radioimmunoassay) but below the level found to be nephrotoxic.

Postoperative Care

It was necessary to maintain significantly higher serum trough CyA levels in our allografts as compared with those for clinical renal transplants. Since CyA is toxic at high levels, serum concentration was measured 3 times a week the first month, biweekly for the next month, and weekly thereafter. If any signs of rejection appeared, the dosage was increased to provide levels over 1,000 ng per milliliter. The most common serious side effect of high CyA levels is nephrotoxicity [4, 13, 18, 20], and at high dosage in combination with other immunosuppressants, hepatotoxicity [4, 5]. Therefore, serum levels of urea nitrogen and creatinine as well as liver enzymes were monitored on the same schedule as CyA levels. A weekly blood cell count was made to ensure that the drug was not causing leukopenia, although this complication is less common than with standard immunosuppression [5].

The first assessment of the allograft was made within the first week, when technical success was determined. Rejection can occur within 2 weeks in an initially successful free flap transplant in the nonimmunosuppressed recipient [22]. Thus, following the initial check, the status of the flap was verified at least twice weekly. Signs indicating rejection include edema, erythema, induration, mottling, vesiculation, ulceration, vascular compromise, necrosis, and eschar formation [6, 8, 9, 22]. In the presence of any of these signs, a punch biopsy specimen was sent for immediate assessment to our consultant pathologist. If histological evidence of rejection was present, therapy was instituted by increasing the CyA to a dosage at

which serum trough levels ran just over 1,000 ng per milliliter. Concurrently, a 3-day course of intramuscular methylprednisolone was given (10 to 15 mg per kilogram of body weight per day), followed by 30 mg of prednisone orally per day, tapered by 5 mg every second day to a maintenance dose of 5 mg per day or the equivalent as intramuscular methylprednisolone.

Assessment of Reinnervation

From previous experiments in our laboratory it is clear that, following ulnar nerve transection at the wrist, a period of at least four months is necessary before the distal tip of the fifth digit becomes reinnervated [24]. Assessment is done at four to eight months, since all skin regions should have innervation, with near complete receptor maturation in the more proximal areas.

To assess the state of reinnervation, each animal was anesthetized and the nerve isolated using microsurgical technique. After opening the connective tissue sheaths, a fascicle was cut free and teased until it contained, when placed on the recording electrode, only 1 or 2 axons. The conduction velocity and the location of the cutaneous receptive field were determined for every isolated axon. When located, the receptive field was carefully mapped under the microscope, using an esthesiometer set to deliver a constant force near the upper limit needed to activate mechanoreceptors. This procedure ensures that freshly reinnervated receptive fields are mapped in a manner comparable to the normal ones. After mapping, the responses of the mechanoreceptive afferent fibers were categorized with respect to receptor modality, submodality, and threshold of activation, by the application of precisely controlled mechanical stimuli.

Experiments and Results

Transplanted Neurovascular Free Flap

The transplanted neurovascular free flap (TNVFF) in the baboon is a modification of the clinically useful digital island flap. The entire skin coverage of the second digit is employed in the design and has the following characteristics: (1) pure sensory innervation (digital nerves), (2) consistent vascular supply (digital artery/dorsal vein), and (3) adequate pedicle length, allowing anastomosis at wrist level. Initial viability was proven in pilot studies, first as an island flap and then as a free flap replanted in situ.

Transplantation between baboons was undertaken (Fig 1). Under general anesthesia, two baboons were placed in the supine position with the right upper limb abducted. The proposed skin incisions were marked, encompassing the entire soft tissue coverage of the digit from the metacarpophalangeal joint to the nail bed. The dissection was begun in the palm of the recipient animal after application of a pneumatic tourniquet. The nerve and vessel sizes, adequate levels of sectioning and repair, pedicle lengths, as well as skin cover dimensions were determined. The proper digital artery to the ulnar side of the second digit was isolated to its origin from the radial component of the single palmar arch, via the common palmar digital artery to the second web space. Arterial size approached 1.0 mm at wrist level. The digital nerves as well as the dorsal branches of the radial nerve to the second digit were separated from adjacent structures. All nerves were tagged with 10–0 sutures to indicate radial and ulnar orientation, thus permitting a more accurate alignment for subsequent nerve repairs. A large dorsal digital vein was freed from the proximal edge of the dorsal flap proximally to the dorsal forearm, where its diameter approached 1.3 mm. The digital nerves were then sectioned and labeled in the midpalm and middorsal hand, respectively. The flap was raised superficially to the tendons in a distal to proximal direction. The artery was clamped with an Acland 2V vessel approximator at the wrist and sectioned distally, while the vein was similarly transected in the distal forearm. Division of the vascular and neural pedicles was done at a level to maintain sufficient length in the recipient limb.

The donor flap was then prepared in a similar fashion, maintaining long neurovascular pedicles to the flap. The flaps were removed from their respective beds and the donor flap transplanted to the recipient. The vascular anastomoses were done with an operating microscope using standard microsurgical technique. Nine to ten simple interrupted stitches of 10–0 nylon on a 75-micron needle were required. The success of the microanastomoses was shown intraoperatively by the patency test. Once the flap was revascularized, the appropriate nerve bundles were aligned and repaired with 3 to 5 simple interrupted stitches. Hemostasis was secured with bipolar cautery and hemoclips prior to suturing the flap into its bed. Determination of postoperative status was performed within the first week posttransplant and twice weekly thereafter.

We have achieved survival of as long as 161 days in a TNVFF thus far with complete healing of the flap to its recipient bed.

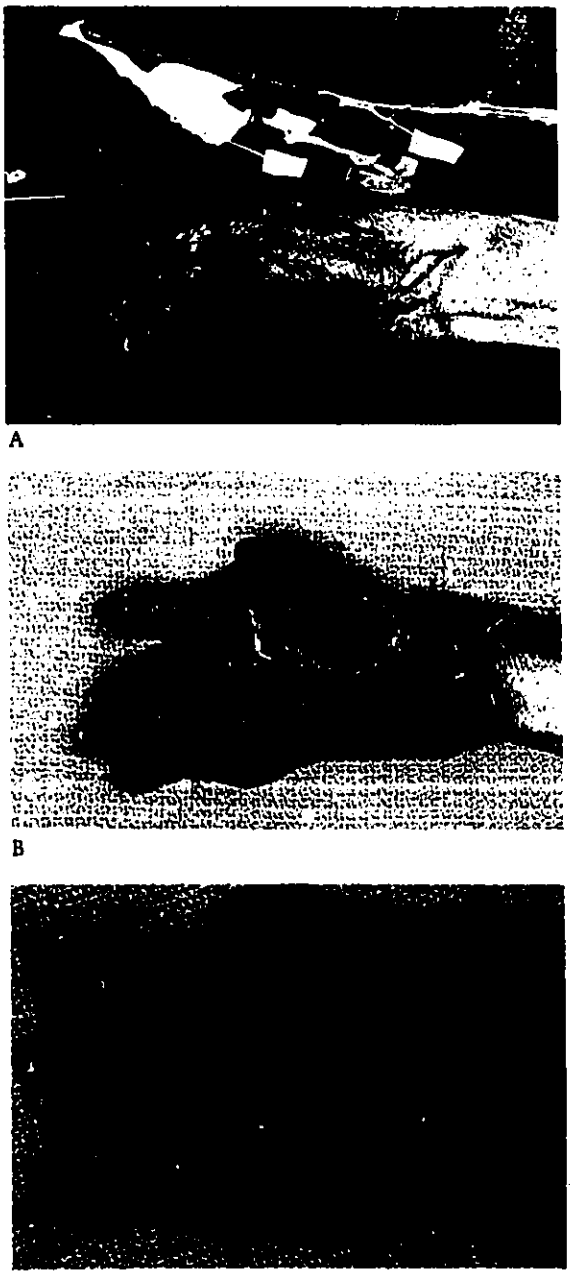

Fig 1. Transplanted neurovascular free flap. (A) Donor neurovascular free flap next to recipient hand prior to excision of recipient flap. (B) TNVFF immediately postoperatively. (C) TNVFF 69 days postoperatively.

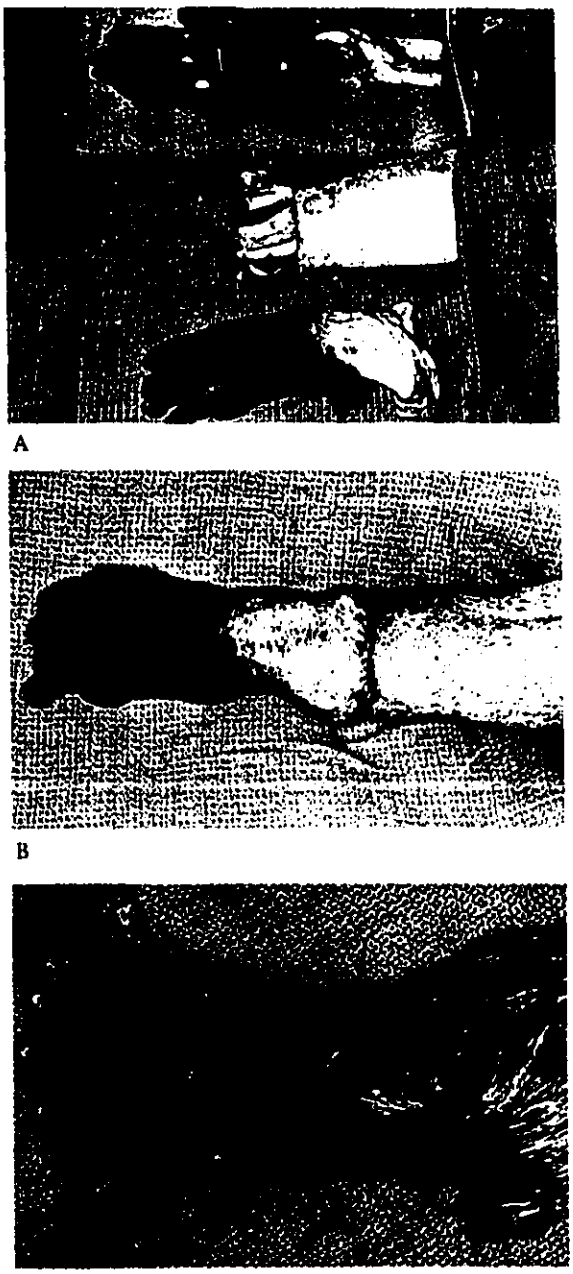

Fig 2. Hand transplant. (A) Top, *recipient hand;* middle, *recipient forearm;* bottom, *donor hand. (B) Transplanted hand, immediately postoperatively. (C) Transplanted hand 124 days postoperatively. Note the hair growth in the proximal end as compared with the immediately postoperative photograph.*

Hand Transplant

A hand transplant was undertaken in the baboon utilizing a surgical method similar to that used in clinical replantation. Using this method permits assessment of the long-term survival of compound tissue with skin, muscle, and bone as well as vascular and neural structures. In addition, it permits eventual neurophysiological studies to evaluate the return of both sensory and motor nerve function.

Prior to the first hand transplant, anatomy of the baboon forearm was reviewed and dissections were performed. Transplantation of the hand at the distal forearm level was undertaken (Fig 2) using the following operative technique: Under general anesthesia, 2 baboons were placed in the supine position with the right upper limb abducted. The proposed skin incision was marked circumferentially on the forearm of the recipient 4 cm proximal to the wrist joint. After inflation of the pneumatic tourniquet, the incisions were carried to subcutaneous tissue. Skin flaps were raised in proximal and distal directions, then all tendons were identified, marked 2 cm from the wrist, and sectioned at the joint. The median, ulnar, and radial nerves were identified, tagged, and sectioned approximately 2 cm from the wrist. The radial artery and cephalic vein were also identified at the same level, where they measured 1.0 to 2.0 mm in diameter. They were clipped with small hemoclips and sectioned. Dissection was then carried deep to the radius and ulna. The periosteum was raised to free the bones at the 6-cm level, where they were divided with an electric saw. Hemostasis was secured with bipolar cautery and hemoclips, following release of the tourniquet. A similar procedure was undertaken in the donor; again tendons were marked 2 cm from the wrist and divided along with the neurovascular structures to maintain maximal length to the donor segment. The bones were sectioned 4 cm from the wrist.

Transplantation was undertaken beginning with bony internal fixation using compression plates. Next, all deep tendons were repaired utilizing mattress sutures in the extensor compartment and the Pulvertaft technique for the flexors. Tension was adjusted by lining up the 2-cm marks on the donor and recipient tendons prior to repair. The vascular anastomoses were undertaken with an operating microscope and standard microsurgical technique. Following excision of redundancy, 10 to 12 simple interrupted stitches of 10–0 nylon on a 75-micron needle were required. Once the hand was revascularized, the appropriate nerves were aligned and repaired with 3 to 5 10–0 nylon simple interrupted stitches. The remaining tendons, except for the flexor digitorum superficialis, were repaired, followed by hemostasis with the bipolar cautery and hemoclips. The success of the microanastomoses was shown intraoperatively by the patency test. Any excess skin was trimmed and the wound closed with 4–0 polyglycolic acid simple interrupted and continuous stitches. Determination of the postoperative status was performed within the first week posttransplant and twice weekly thereafter.

One of our hand transplants is currently in excellent condition at 150 days. Long-term survival is becoming progressively more definite as experience is acquired with these transplants. Motor and sensory nerve function will be assessed within the coming months.

Discussion

These studies bring us a step closer to the day when tissue transplantation for reconstructive purposes may provide solutions for patients with irreparable problems. However, certain specific challenges remain as related to characteristics of immune modulation, the individual patient, and the allograft tissue. Patients in whom reconstructive transplantation would be considered represent a young population with a long life expectancy. This is in contrast to the usual patient requiring vital organ transplantation. Consequently, they will require life-long immunosuppression, in its present form, for the maintenance of their allografts. CyA is the first immunosuppressant to permit success in tissues previously found impossible to transplant. Unfortunately, the use of this drug is associated with several minor and a few major side effects. Whereas it is valid to use powerful immunosuppressants with potential toxicity for life-saving solid organ transplantation, the acceptability of such treatment differs markedly in those patients for whom the goal is enhancement of the quality of life. However, future introduction of CyA derivatives with acceptable side effects may provide the opportunity to begin long awaited reconstruction utilizing tissue transplants.

Another factor that may affect the outcome of this type of surgical procedure is the health of the patient. Certainly, a young, healthy individual whose only medical problem is that of a limb amputation would be expected to do very well following any surgical procedure. The immune system will not be depressed, but rather quite active. This feature may or may not be helpful in the outcome of the transplant, depend-

ing on the mode of action of future immunosuppressants. If it becomes possible to select drugs that function best for patients with normally active immune systems, this characteristic may result in an increased, rather than decreased, success rate. In contrast, in patients with renal, hepatic, or cardiac failure there is already a degree of immunosuppression prior to transplantation, and the previously acquired patient management skills may not be applicable to a new form of clinical transplantation.

Also in question is the immunogenicity of transplanted skin as well as the effect of compound tissue. Certainly, skin is expected to require higher degrees of immunosuppression for survival. The questions remain whether this requirement may become a limiting factor in the clinical application of reconstructive transplantation and whether we may need to await the development of a less toxic immunosuppressant. The effect of using compound tissue allografts, when compared with individual organs, is unknown.

These questions must be weighed prior to any attempts at reconstructive transplantation in man. Although it is tempting to begin clinical hand transplants, three factors must be carefully considered: (1) functional sensory and motor reinnervation must be proven, (2) extensive laboratory experience in this type of reconstructive transplantation program along with the support of a strong clinical transplantation service must be available, and (3) the present necessity to use a powerful immunosuppressant with serious side effects in combination with steroids indicates the need for continued research into improving the modalities available for immune modulation.

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ORIGINAL COMMUNICATIONS

Tissue transplants in primates for upper extremity reconstruction: A preliminary report

Recent advances in clinical transplantation surgery suggest that hand transplantation is no longer an unrealistic expectation. However, two questions must be answered. Can composite tissue transplants survive in a primate species? Does the required neural reinnervation occur under immunosuppression? Four hand transplants and seven neurovascular free flap transplants were done in baboons immunosuppressed with Cyclosporin A and steroids (methylprednisolone). Long-term survival occurred in nine. Electrophysiologic tests of sensory axons revealed reinnervation of transplanted skin as evidenced by well-defined, low threshold receptive fields in the donor tissue. Reinnervation of donor muscle was demonstrated by motor unit recruitment in stepwise fashion after electrical stimulation of the recipient's median and ulnar nerves. Afferent fibers serving the donor's joints and muscle spindles were also observed. (J HAND SURG 11A:1-8, 1986.)

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Despite the efficacy of replantation surgery and free tissue transfers, a significant group of patients exists with devastating injuries that defy reconstruction. If feasible, tissue transplantation would be the ideal solution for these individuals. The recent introduction of Cyclosporin A (CyA) has led to high success rates in conventional transplantation of kidney,[1,2] liver,[1] bone marrow,[3] and heart;[4] furthermore, it has permitted the transplantation of highly antigenic tissues including the lung.[5] Experimentally, this drug has proved to be effective for transplantation of nerve,[6,7] muscle,[8,9] bone,[10] and skin.[11-14] The relative safety and excellent specificity of CyA have rekindled the hope that reconstructive transplantation may become a clinical reality.

Unfortunately, major differences exist between parenchymal organ transplantation and transplantation of those tissues required for upper extremity reconstruction. A hand transplant consists of multiple tissues with varying degrees of antigenicity; skin particularly presents an extreme challenge to the immune system.[15-17] In addition, unlike renal and cardiac transplants that function independent of direct neural regulation, allo-

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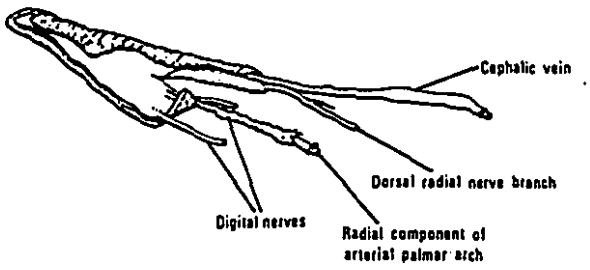

Fig. 1A. Transplanted neurovascular free flaps.

grafted digits and hands would require reinnervation to be useful. Since CyA may block the response to antigenic stimulation through molecular mechanisms not as yet clearly defined, it may affect other membrane recognition processes including those required for the recipient's axons to identify donor sensory receptors and motor end plates.[18]

This study addressed the issues of long-term survival of transplanted composite tissues in a primate species and its functional reinnervation under CyA immunosuppression. Two experimental models in the baboon were designed for this purpose. These models are described below. Evidence for reinnervation of the transplanted tissues is also presented.

Material and methods

Experimental animal. The criteria for selecting the experimental animal included the following: (1) anatomic similarity of the hand to that of man including comparable sensory receptors and nerve pathways, (2) neurovascular structures of sufficient size to permit microsurgical repair and neurophysiologic recording, (3) a species high in the phylogenetic scale, and (4) a reliable source of these animals. The baboon, specifically *Papio anubis*, was chosen. Since this species had previously been used in our laboratory for the study of reinnervation after peripheral nerve repair, established techniques and protocols were available.[19] Young, female, adult baboons weighing between 9 and 14 kg were used, since they are far easier to handle than the larger, more aggressive males. The animals were tissue typed for the major histocompatibility antigens in a manner similar to that used for human typing. Microcytotoxicity testing[20] showed that donor-recipient pairs 16 and 20, 18 and 19, and 18 and 21 had two chromosomal differences. Pair 16 and 17 shared one allele, but had a high probability of being unrelated. The rejection episodes seen in the remaining recipients (those not tissue typed) indicate that genetic dissimilarities existed in all donor-recipient pairs.

Immunosuppression. Beginning 4 days before surgery and continuing for the length of each study, CyA was administered by intramuscular injections twice daily (20 mg/kg, dissolved in Miglyol 812 and absolute ethanol as per Sandoz). We attribute the need for this high dosage to the Miglyol 812 preparation and its intramuscular deposition. Dosages were adjusted to maintain 12-hour serum trough levels between 800 and 1000 ng/ml as monitored by radioimmunoassay performed at room temperature. Through experience with the initial transplants, supplemental methylprednisolone was found necessary beginning at the time of surgery. The present steroid protocol consists of 125 mg/day for 3 days, followed by a tapering regimen starting at 25.6 mg/day divided into two doses, decreasing by 4.0 to 4.4 mg on alternate days to a maintenance dose of 4.4 mg once daily.

Perioperative management. Sterile surgical technique was used at all times. General anesthesia was maintained with intravenous sodium pentobarbital after initial tranquilization with a mixture of ketamine and xylazine (5.25 mg and 0.45 mg/kg, respectively). Physiologic parameters (urinary output, heart rate, rectal temperature, and expired carbon dioxide) were monitored. Electrolytes and fluids were administered as needed to maintain homeostasis. Pure oxygen was administered via an endotracheal tube, and ventilation was self-controlled. A single dose of heparin was given intravenously (70 IU/kg) to both donor and recipient during surgery. After appropriate dressings, a custom-made thermoplastic splint was applied to protect the operated limb. Each animal received a 2-week course of prophylactic intramuscular antibiotics initiated 1 day before surgery (procaine and benzathine penicillin, 11,000 IU/kg/day of each and netilmicin, 50 mg twice daily). Levorphanol tartrate (1 mg twice daily) analgesia was maintained for 5 days. Oral acetylsalicylic acid (75 to 100 mg/day) was given for approximately 10 days after surgery.

Postoperative care. Very high CyA levels were found to be necessary for long-term survival of these transplants. Since CyA is nephrotoxic at high levels and hepatotoxic when combined with other immunosuppressants, careful monitoring was required.[1-4] Serum levels of CyA, urea nitrogen, creatinine, and liver enzymes were determined from blood samples drawn three times per week for the first month, twice weekly for the next month, and thereafter according to the stability of the animals. A complete blood cell count was usually performed once a week to monitor for leukopenia. Although this complication is uncommon with CyA,[2] it can occur with long-term administration of

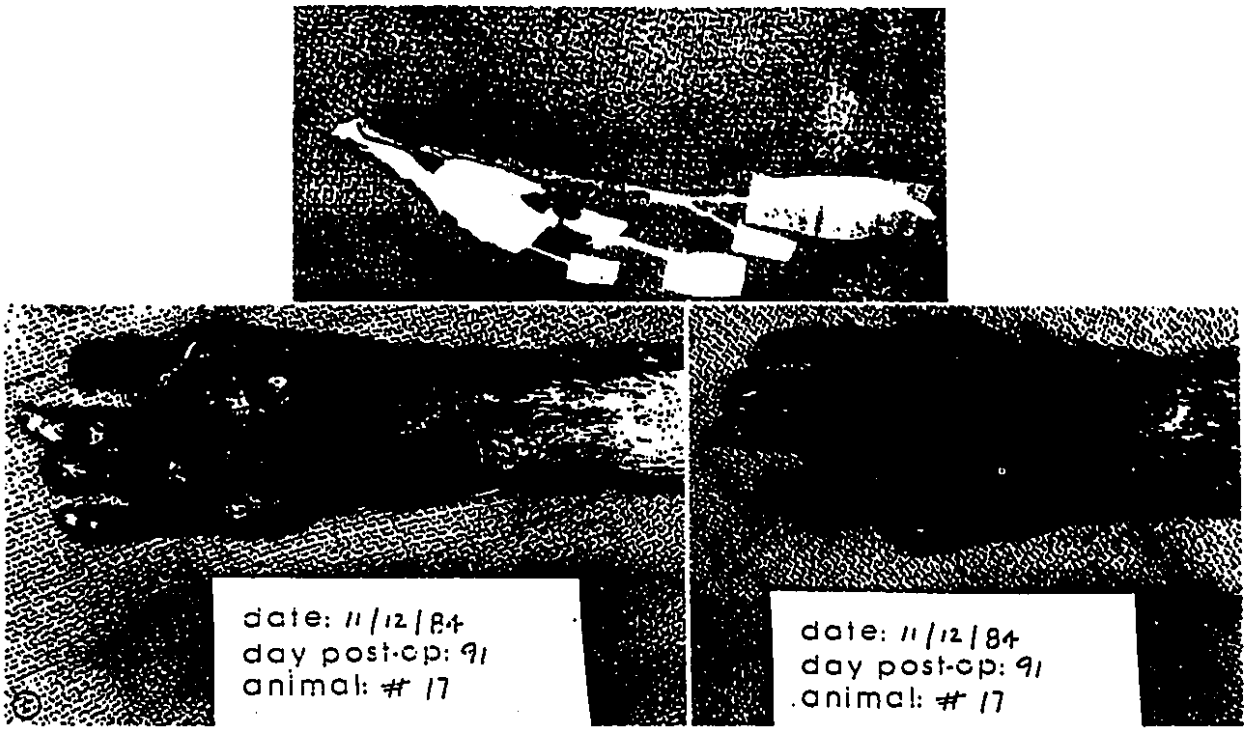

Fig. 1B-D. B, Skin of index digit isolated on its neurovascular structures. **C-D,** Complete survival of the transplanted flap at 91 days; note dorsal hair growth.

steroids. Technical success was determined at the first dressing change on the third postoperative day. Since rejection of a free flap in a nonimmunosuppressed recipient is known to occur within 2 weeks in certain experimental models,[21] the allografts were examined at least twice a week. Expected signs of rejection included edema, erythema, induration, mottling, vesiculation, ulceration, vascular compromise, necrosis, and eschar formation.[21-24] In the presence of clinical signs of rejection, the CyA dosage was adjusted to maintain 12-hour serum trough levels just over 1000 ng/ml, and the methylprednisolone was restarted at the beginning of the dosage regimen. Punch biopsies were sent for histologic assessment, but differentiation of rejection from infection was extremely difficult. With experience, clinical assessment proved to be an accurate method of determining early rejection.

Experiments

Neurovascular free flap transplant. The neurovascular free flap (NVFF) permitted the transplantation of the entire skin coverage of the second digit (Fig. 1). Sensory innervation was provided by two median-derived digital nerves and small branches of the dorsal radial nerve. The digital arteries were dissected back to the radial component of the palmar arch, and the dorsal veins were traced back to the cephalic vein used to drain it. These long vascular pedicles permitted vascular anastomoses at wrist level, while the nerves were repaired in the mid-palm and on the dorsum.

Complete hand transplant. The complete hand transplant was designed to assess survival and function of multiple tissues (Fig. 2). The technique used in the transplantation of four hands was similar to clinical replantation of a distal forearm amputation. After isolation of essential structures, the hands of both animals were amputated and transplanted to their respective recipient stumps.[25] The tendons in the recipient were marked 4 cm from the wrist, while those of the donor at 2 cm. This was done to adjust for the 2 cm bone shortening present when the donor radius and ulna are sectioned 4 cm from the wrist and the recipient radius and ulna at 6 cm. During tendon repair, these marks are used to reestablish correct tension.

Results

NVFF transplant. All seven of the transplanted NVFFs (animals 5, 11, 15, 17, 19, 20, and 21) were

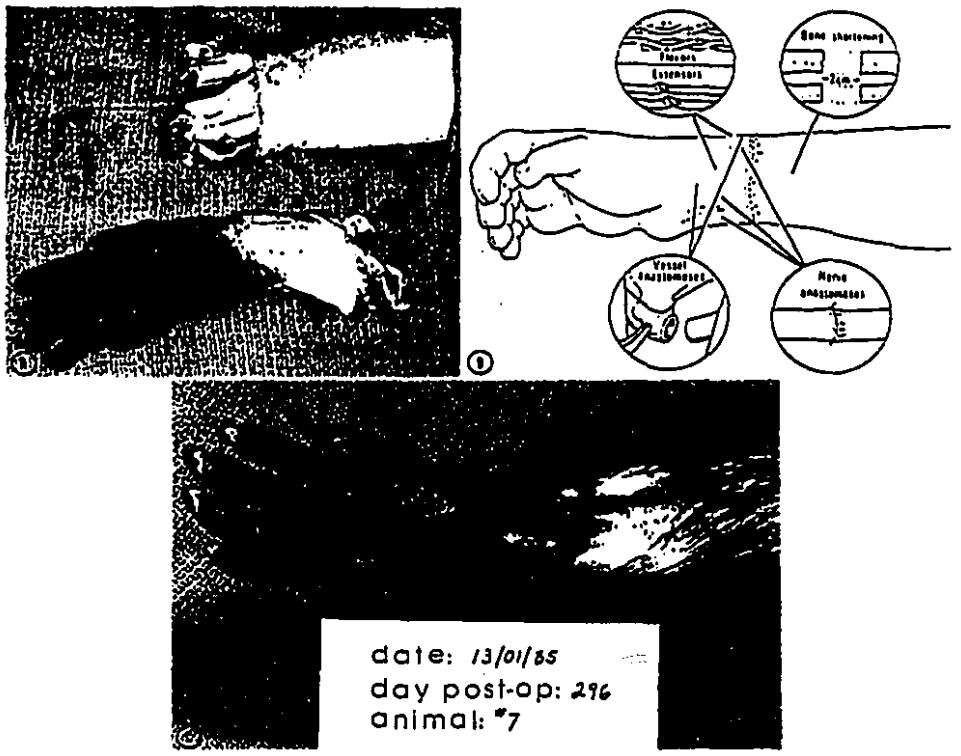

Fig. 2. Hand transplant. A, The donor hand and recipient forearm. B, Schematic representation of surgical technique. C, Survival of the transplanted hand at 296 days.

technically successful (Table I). However, the first three transplants developed signs of rejection including edema, vesiculation, ulceration, and epidermal sloughing. In animal No. 5, the problem was attributed to insufficient preoperative CyA treatment (1 day), inadequate CyA serum trough levels (400 ng/ml), and delayed administration of steroids (methylprednisolone on day 14). Surprisingly, the flap was salvaged, and only the distal portion underwent subsequent episodes of epidermal breakdown and healing. At 161 days, the sensory nerves were tested electrophysiologically[19, 25] to assess the function of single axons. It was clear that the reinnervation of the nonscarred regions of the flap had occurred. Low threshold, well-defined receptive fields of both slowly and rapidly adapting receptor classes were observed in both glabrous and hairy skin. The axonal conduction velocities were slower than normal, and the thresholds for mechanical stimulation of the receptive fields were elevated above those found in normal skin.

An episode of limited rejection of the second (No. 11) and third (No. 15) NVFFs was attributed to a gradual reduction of the maintenance dose of CyA. The second transplant showed some evidence of epidermal instability over a period of approximately 6 weeks, but rejection was successfully reversed, and electrophysiological testing was performed at 211 days. The results demonstrated that reinnervation had occurred within the hairy skin as evidenced by low threshold, well-defined receptive fields of both receptor classes. However, the heavily scarred glabrous skin on the palmar aspect of the flap showed little evidence of reinnervation. The third NVFF remained unstable after the initial rejection episode, with frequent loss of dry tissue from the glabrous epidermal surface. Electrophysiologic testing on day 147 showed a large number of functional cutaneous mechanoreceptors with well-defined receptive fields of both slowly and rapidly adapting receptor classes.

Complete hand transplant. Four hand transplants (animals 7, 9, 12, and 13) were performed. All were technically successful (Table I). Two of these survived long-term, with animal No. 7 in excellent condition at 304 days after surgery. The other (animal No. 13) was tested for neural function at day 188 after surgery, and

Table I. Summary of transplant results

Animal	*Procedures*	*Rejection*	*Status*
5	TNVFF	Early, 4 weeks' duration	Distal: episodes of breakdown and healing Proximal: very good Sensory nerve testing, 161 days after surgery
7	HT	None	Excellent, 304 days after surgery
9	HT	Hyperacute rejection	Terminated, 26 days after surgery
11	TNVFF	At approximately 3½ mo, 6 weeks' duration	Very good Sensory nerve testing, 211 days after surgery
12	HT	At approximately 1½ mo, irreversible	Terminated, 71 days after surgery
13	HT	At approximately 2 mo, 4 weeks' duration	Good Sensory and motor nerve testing, 188 days after surgery
15	TNVFF	At approximately 2 mo, chronic	Unstable Sensory nerve testing, 147 days after surgery
17	TNVFF	None	Excellent, 132 days after surgery
19	TNVFF	None	Excellent, 59 days after surgery
20	TNVFF	None	Excellent, 105 days after surgery
21	TNVFF	None	Excellent, 87 days after surgery

Legend: TNVFF, transplanted neurovascular free flap; HT, hand transplant.

the hand was harvested for histologic examination. Both had completely healed incisions and hair growth. The other two hand transplants (animals 9 and 12) underwent rejection, with animal No. 9 experiencing hyperacute rejection. At completion of the transplant procedure in this animal, a purplish discoloration was present at the edge of the allograft. Subsequently, deterioration progressed relentlessly over 26 days with marked edema and vesiculation causing glabrous epidermal sloughing over the entire palm and fingers. Infection compromised the remaining dermis, and sudden clinical necrosis of the digits occurred. Rejection of the hand in animal No. 12 was due to an inadvertent iatrogenic reduction in the CyA maintenance doses followed by an infection that prevented salvage.

The electrophysiologic tests of the allografted hand of animal No. 13 showed that both allografted skin and muscle were reinnervated. Despite an early severe rejection episode, both slowly and rapidly adapting cutaneous mechanoreceptors were observed to have low threshold receptive fields in both hairy and glabrous skin. Joint and muscle spindle afferents were also observed. By electrically stimulating separate fascicles of the median and ulnar nerves, it was possible to demonstrate motor unit recruitment of the thenar and other intrinsic muscles in a characteristic step pattern, indicating that each muscle had multiple motor units serving it.

As shown in Fig. 3, *A* (representing a location on the palm of the transplant in animal No. 13), neural activity arising from a rapidly adapting mechanoreceptor produced increasing number of action potentials to increasing intensities of vibratory stimulation. The minimum stimulus amplitude required to produce one action potential per vibration changed as a function of the frequency of the stimulus. This curve, known as the tuning curve, had a shape comparable with that found in normal skin. The initial recordings show that the average threshold for the cutaneous, rapidly adapting mechanoreceptors was higher than normal.

The responses of a slowly adapting mechanoreceptor located on the palm of the transplant in animal No. 13 is shown in Fig. 3, *B*. The graph shows the increasing number of impulses per second that were generated by increasing intensities of skin indentation. Although the response is similar to that seen in normal skin, the rate of adaptation to the steady stimulus appears to occur more abruptly than normal, and thresholds again appeared to be higher (0.5 gm) than those found in normal skin (0.1 gm). By comparing transplants with a rejection episode to those with little or no rejection, it was apparent that many of the abnormalities correlated with the degree of rejection. Those axons serving tissue with minimal rejection had more normal response properties.

Thus, the initial assessment of these transplants shows the presence of reinnervation with the reap-

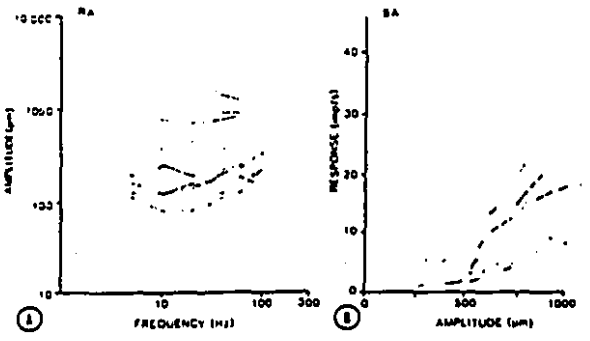

Fig. 3. A, The rapidly adapting afferent fibers could be entrained to vibratory stimuli over a range of frequencies. Thresholds varied in a characteristic manner with frequency thereby producing tuning curves. These curves were within normal limits, but tended to be above normal mean values. B, The slowly adapting afferent fibers responded with trains of impulses during sustained deformation of the skin. The slopes of the stimulus response curves were within normal ranges, but the thresholds were slightly elevated.

pearance of the two major classes of cutaneous mechanoreceptors. In a sample of 115 conduction velocity measurements, the reinnervated axons displayed conduction velocities significantly lower than normal, but within the population of those serving control NVFFs in the presence of CyA (Fig. 4). The data available clearly demonstrate that reinnervation occurs for all major structures in the transplant and that these structures provide the substrate for the appropriate neural responses to be sent to the central nervous system.

DISCUSSION

Interest in limb transplantation was initiated by the legend of Cosmas and Damian,[26] two brothers who donated their medical talents to heal the sick. Martyred near the end of the third century, they reappeared nearly three centuries later at the Basilica of Saints Cosmas and Damian in Rome to perform the miracle of the black leg. According to legend, they replaced the cancerous limb of a devoted follower "while he slept" with that of a recently deceased Ethiopian moor. Until 30 years ago, little hope existed for modern man to emulate this wondrous deed. In 1954, the first successful renal transplantation between identical twins was reported.[27] In the 1960s, 6-mercaptopurine and azathioprine were introduced, and successful renal transplants followed. Prednisone was soon added to the azathioprine, leading to an improved success rate through the use of two agents with differing targets of immunosuppression.[28]

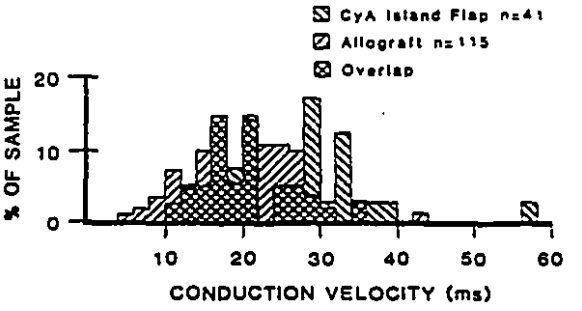

Fig. 4. Conduction velocities of the fibers innervating the transplanted hairy and glabrous tissues were significantly reduced below normal, but remained with the population representing those serving replanted NVFFs in the presence of CyA.

Experimental whole limb transplants were subsequently achieved with methods impracticable in man. Schwind[29] used parabiosis before 2 weeks of age in rats to successfully transfer a limb for a 14-day period. Lapchinsky[30] achieved immunologic tolerance in a dog through complete exchange transfusion from the donor when the recipient was 9 days old. Nine months later, a limb was transplanted and reported to have survived at least 2 months. Attempts have also been made to induce tolerance in adult transplant recipients. Poole et al.[31] found it possible to achieve prolonged rat limb allograft survival in recipients having had a previous antiserum-enhanced kidney allograft. It was noted that the longer the time elapsed between the kidney and the limb allograft, the longer the limb survived.

Several other authors undertook adult animal limb transplantation with various combinations of immunosuppressive drugs other than CyA. Goldwyn et al.[32] used 6-mercaptopurine and azathioprine in dogs. Although these drugs slightly prolonged survival, they did not prevent rejection. In some cases, the drug regimen caused fatal systemic side effects. Doi[33] noted similar results when he attempted to transplant rat limbs. Azathioprine- and prednisolone-treated rats, although showing some increase in allograft survival, succumbed to the side effects of the immunosuppressants. None of the other treatment groups studied (6-mercaptopurine and prednisolone, azathioprine, prednisolone, and 6-mercaptopurine) achieved prolonged survival. The only study with prolonged survival before the advent of CyA was that of Lance et al.[10] who transplanted canine hind limbs between unrelated registered beagles using various potent combinations of antilymphocyte serum, azathioprine, hydrocortisone acetate,

thymectomy, splenectomy, exchange transfusion, and splenic cell suspension. Although significant prolongation of allograft survival occurred, the dogs receiving continuous immunosuppression had severe side effects including pancytopenia, wound infection, and systemic sepsis. Greater success occurred in three animals receiving a short-term course of massive immunosuppressive drug therapy, with or without splenectomy or thymectomy, and followed by induction of immune tolerance from donor splenic cells or exchange transfusion. Although these dogs ran a somewhat unstable course, long-term survival was achieved, one rejected on day 200, and the other two survived beyond 60 and 300 days, respectively.

In 1982, Black et al.[32] published the first data on survival of adult rat limb transplants with CyA immunosuppression. The final results followed a year later.[33] Brown Norway/Lewis limbs were transplanted to Lewis rats. Animals that received 20 days of subcutaneous CyA at 25 mg/kg/day showed a dramatically increased transplant survival, with at least one animal keeping its allografted limb beyond 225 days. Fritz et al.[33, 34] also successfully transplanted adult rat limbs. The hind limbs of ACI-strain rats were heterotopically transplanted to the backs of Lewis rats representing "a very strong antigenic mismatch." The CyA-treated animals, receiving 10 mg/kg/day subcutaneously for 7 days, 21 days, or continuously, performed significantly better than the controls. No CyA-treated animals developed strong clinical signs of rejection, and nine had no histologic evidence of rejection. Antibody testing revealed a strong positive response in all untreated animals, but none in the long-term CyA treatment group.

Kim et al.[35] recently published the results of another transplantation study of adult rat limbs. Hind limbs in Buffalo rats were allografted orthotopically to Lewis recipients. Six groups were studied including a replant control, a nondrug control, and two older immunosuppressive regimes. As expected, the replants showed edema immediately after surgery and subsequent denervation atrophy, but otherwise remained intact. Rejection as judged by clinical signs occurred around 6 days in the controls and about 7½ days with the traditional immunosuppressive regimens. In contrast, the CyA-treated rats behaved similarly to the replant group, with signs of rejection appearing only after CyA therapy was stopped. The literature is clear on two points: (1) CyA is strikingly better than previously available procedures for immunosuppression in composite tissue transplants, and (2) no previous studies of functional reinnervation exist for composite tissues.

From these reports the need for transplants in primates and to determine the capacity for functional reinnervation are obvious. Of our 11 long-term allografts, five (one hand, four NVFFs) have had prolonged survival in excellent clinical condition, three have undergone reversible rejection episodes, one has remained in a state of chronic but partly controllable rejection, and two have had uncontrollable rejection. Of the six rejection episodes, five occurred early in the study and might have been prevented with the knowledge and protocols presently in use. Despite the problems encountered, we have demonstrated that in nine of 11 cases, prolonged survival of highly antigenic allografts was possible in a primate.

The initial electrophysiologic tests have proved that both sensory and motor reinnervation will occur across major primate histocompatibility barriers in the presence of high serum levels of CyA. In both the hand transplant and the NVFF, low threshold, well-defined receptive fields of both slowly and rapidly adapting cutaneous mechanoreceptors were identified in glabrous and hairy skin. In addition to observing reinnervation of skin, muscle spindles and joint afferents were also electrophysiologically characterized. Contraction of the thenar and other intrinsic muscles was observed after nerve stimulation. Motor unit recruitment was seen in characteristic well-defined steps.

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