ALLOGENEIC TRANSPLANTATION of the RADIAL SIDE of the HAND in the RHESUS MONKEY

Technical, functional and immunological aspects

ALLOGENE TRANSPLANTATIE van de RADIAIRE ZIJDE van de HAND bij de RHESUSAAP

Technische, functionele en immunologische aspecten

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 22 MEI 1991 OM 13.45 UUR

DOOR

STEVEN ERIC RUDEN HOVIUS

geboren te Punta Cardon, Venezuela

EN

HIERONYMUS PETRUS JOSEPHUS DIEUDONNÉ STEVENS

geboren te 's Gravenhage, Nederland

universiteits
Drukkerij

1991

Steven   ter nagedachtenis aan mijn vader, aan mijn moeder
          aan Annerieke en Willemijn, Wouter, Jeroen en Guus

Jeroen   aan mijn ouders, Karin en naar ik hoop ons nageslacht

----

PREFACE

As this is the era of transplantation it is inevitable that the field of allogeneic transplantation for the reconstruction of the upper extremity is explored also.

This double-thesis deals with a number of aspects concerning allogeneic transplantation of the radial side of the hand in a rhesus monkey model. In the introduction the reasons for investigating the possibility of hand transplantation from one individual to another are clarified. In particular, the reasons why experiments in a nonhuman primate model were preferred, are explained.

Should hand transplantation in man ever be performed, three major questions will always be foremost: is allogeneic transplantation of such a composite tissue allograft technically feasible, can allograft acceptance by the host be established, and if so will sensory and functional recovery occur? These questions inspired the authors to perform the experimental work presented. An attempt was made to integrate the major multi-disciplinary facets, clinically as well as preclinically. S.E.R. Hovius focussed on the technical and functional aspects of transplantation of this composite tissue allograft. H.P.J.D. Stevens examined the immunological aspects of this subject, and new ways to improve the immunosuppressive regimen for transplantation.

Prior to the rationale of the experiments an introduction with regard to these aspects is presented.

ABBREVIATIONS

AMA	Anti-Mouse-Antibody
CD	Cluster of Differentiation
CGR	Complete Graft Rejection
CMAP	Compound Motor Action Potential
CSAP	Compound Sensory Action Potential
CTA	Composite Tissue Allograft
CyA	Cyclosporine A
DAF	Di-Adreson F-aquosum
ELISA	Enzyme-Linked Immunosorbent Assay
EMG	Electromyography
FACS	Fluorescent Activated Cell-Sorter
HA	Hematoxylin-Azafloxin
HLA	Human Leukocyte Antigen
IFN-γ	Interferon-gamma
IL	Interleukin
LDF	Laser Doppler Flowmeter
MAb	Monoclonal Antibody
MHC	Major Hostocompatibility Complex
PTLP	Post-Transplantation Lymphoproliferative
PU	Perfusion Unit
RIA	Radioimmuno Assay
SD	Standard Deviation
STLV	Simian T-Leukemia Virus
TNF-α	Tumor Necrosis Factor-alpha

CONTENTS

CHAPTER III: ALLOGENEIC PARTIAL HAND TRANSPLANTATION IN THE RHESUS MONKEY

STUDY:

CHAPTER IV: NEW APPROACHES TO OPTIMIZE IMMUNOSUPPRESSIVE THERAPY

STUDY:

S.E.R. Hovius presents chapter I, sections A and C; chapter II, studies A, B and C; chapter III, studies A and C; chapter V and hoodfstuk VI. He has contributed to chapter III, studies B and D; and chapter IV, study B.

H.P.J.D. Stevens presents chapter I, sections B and C; chapter II, studies D and E; chapter III, studies B, D and E; chapter IV, studies A and B; chapter V and hoodfstuk VI. He has contributed to chapter II, studies B and C; and chapter III, studies A and C.

CHAPTER I: INTRODUCTION
SECTION A: Technical and functional aspects

S.E.R. Hovius

There was an ape in the days that were earlier
centuries passed and his hair became curlier
centuries more gave a thumb to his wrist;
then he was man - and a positivist.
[Mortimer Collins (1827-1876)]

A.1   Hand reconstruction and replantation surgery
A.1.1 Hand reconstruction: a prospective view

The ultimate goal of the subject of this double-thesis is to perform allogeneic human hand transplantation. We would like to illustrate the problems involved with the following scenario, which represents some speculations on the future of allogeneic hand transplantation.

Scenario of the 21st century.
"The 18-year old P.J. lost his right hand just proximal to the wrist whilst cleaning an automatic gripping device in a large factory. As the control switch accidently was touched, his right hand was caught in the machine and crushed. He was right handed.

Following first aid at the factory, he was sent directly to a large upper extremity trauma unit. Unfortunately replantation proved to be impossible due to severe crushing of the hand with destruction of nearly all of the finger joints. Using a small dynamic Magnetic Resonance Imaging (MRI) machine, the exact extent of the non-vital tissues of his forearm could be assessed. At operation the non-vital tissues were removed. Debridement was found to be adequate following a quick peroperative assessment with the portable MRI machine. All clean tendons, nerves and vessel-ends were sealed off, with a biocompatible polymer containing an antifibrous tissue agent to prevent local fibrosis. As a cadaver transplant was planned in the near future, the forearm bones could be shortened and the wound adequatly covered with normal skin.

Three months later Eurotransplant called to say that a donor right hand of a young man was available, and that it matched well with the Major Histocompatibility Complex (MHC) - antigens of 18-year old P.J. Immediately a surgical team flew in to prepare the cadaver forearm for transplantation. Following transection of the transplant at the proximal forearm, the vessels were attached to a portable Fluosol-extracorporeal perfusor containing antibodies to desensitize the transplant. The transplant was placed in a continuously chilled (4°C) box, ready for transportation.

In the meantime P.J. was admitted to the upper extremity unit. In view of the pending hand transplantation infusions were administered with a new generation of highly specific, immunosuppressive agents. His right forearm stump was operated upon. Fibrosis was found to be minimal. Nerve, vessel and tendon ends were dissected and prepared for anastomosis. Following arrival of the transplant it was matched for length, of bones, tendons, nerves, vessels and skin, with the acceptor site. The bones were fixed with a biodegradable intramedullary memory device reacting to the external forces, while bone healing took place. Tendons and nerves were connected with fibrin glue. Proximal and distal to the nerve anastomoses special stimulating mini-electrodes were placed as well as a toroid registering magnetic fields to qualify and quantify nerve regeneration. Also small microcapsules releasing nerve growth factors were left near the nerve anastomosis to enhance initial axon ingrowth. The nerve ends could be meticulously matched with staining techniques to identify the fascicles for various motor and sensory qualities. A biodegradable microvascular coupler was used to anastomose the vessels. At different sites around small vessels and in the dermo-epidermal junction sensors were placed to assess the onset of infiltration specific for rejection and to permit early treatment. Following skin closure P.J. was taken care of in a special nursing unit equipped with continuous microvascular monitoring devices to detect vascular compromise or changes in blood viscosity and coagulation. With the aid of these monitoring systems, vascular complications were limited to a minimum. Early warning of initial rejection by the aforementioned sensors located in the dermo-epidermal junction of the allograft, made it possible to cope with immunological complications by altering the administration of the immunosuppressive agents on demand. The drug dosage was regulated by a subcutaneous pump in the subclavicular space, installed at completion of the operation.

Two weeks later P.J. was discharged. An intensive hand therapy programme, including dynamic splintage and nerve stimulation followed. Regular biopsies were taken to check the warning system of the sensors in the dermo-epidermal junction for early rejection infiltrate. At three months a patchy epidermiolysis occurred, which could be adquatly treated with genetically engineered antibodies. At six months good sensory recovery was encountered as well as full flexion and extension. At twelve months postoperatively P.J. had already enrolled in a computer programming study. He was playing squash using his right hand."

Writing this story, the author was inspired by an article of W.W. Shaw[1].

A.1.2 Hand reconstruction: a retrospective view

The reconstruction of the hand was recorded as early as the fourth century, when Saint Julius replanted a left thumb in a workers hand, which had just been amputated by an axe. This feat is represented in a fresco of the 15th century in the Saint Julius Basilica near Milan[2]. Apart from the aforementioned miracle little is known about hand reconstruction in those days. The Middle Ages and the Renaissance period were devoted to the development of prosthetic substitution. Ambroise Paré (1509-1590) in particular designed hand and arm prostheses whose principles are still valid today[3].

Until the mid-nineteenth century no known attempts at hand reconstruction are reported. In view of the scope of this thesis, attention will mainly be concentrated on the severe injured hand with emphasis on the reconstruction of one or more missing digits. A literature review reveals that most efforts in the past were devoted to thumb reconstruction as this element was long recognized as the master digit of the hand. For instance Huguier in France performed a successful phalangization of the first metacarpal in 1852[4,5]. After six weeks the patient was reported to have been discharged from hospital and able to spontaneously approximate the first metacarpal to the second, he could grasp a book forcefully. Guermonprez (1849 - 1932), a French surgeon, was probably the first to write a book on Hand Surgery, which was published in 1895. He performed digital transposition, first on cadavers and then on a macaque monkey. His first clinical transposition was carried out in 1885 when remnants of the middle finger were transposed to reconstruct a thumb in a 13-year-old boy whose thumb and index finger were missing[6]. Twenty-five years following this operation the patient was carrying weights and driving his horse-cart while using his transferred finger remnant perfectly as a thumb. Guermonprez is considered to be the forefather of pollicization.

While the French used the remnants of the injured hand to reconstruct the thumb, Nicoladoni (1847 - 1902), an Austrian surgeon, conceived new and different methods. In 1891 he reconstructed a degloving injury of the right thumb with a tubed pedicle flap from the pectoral region[7]. Nicoladoni also suggested the use of bonegrafts from the tibia to lengthen short stumps before coverage with a tubed pedicle flap. In 1898 he performed a pedicled transfer from the second toe to the amputated thumb of a 5-year old boy. Sixteen days later the skin pedicle was divided. Even though the distal phalanx became necrotic, the functional result was reported to be good[8]. Following the suggestions of Nicoladoni, the British surgeon Joyce described a digital transfer of the ring finger of the left hand to the stump of the amputated right thumb in 1918[9].

In the Netherlands Laméris published, in 1909[10], a successful replacement of the amputated left thumb with the second toe of the right foot in a 12-year old boy. The blue

discoloration which occurred postoperatively was relieved with leeches. This procedure is still used today to treat venous congestion in flaps.

One of the pioneers of modern plastic surgery was the Dutchman J.F.S. Esser (1877 - 1946). His influence on the development of plastic and reconstructive surgery is eloquently described in the thesis of B. Haeseker[11]. Esser carried out fourteen toe-to-hand transfers. In 1916 he even transplanted four toes with a part of their corresponding metatarsals to reconstruct four lost fingers including their metacarpophalangeal joints[12]. A Hungarian soldier had lost his four fingers as the result of an exploding handgrenade, his thumb was scarred and immobile. Following excision of the scarred tissue of his right hand the right foot was brought to his hand, and incised dorsally. Tendons and bones were transected at different levels. The plantar side of the foot served as vascular pedicle. All structures were meticulously adapted, periosteum, fascia, tendons, tendon sheaths and nerves by suturing adjacent tissue. The first night he fell out of bed with his heavy uncomfortable cast. Fortunately only a slight wound dehiscence occurred, which healed well. The pedicle was stepwise divided in the fourth and fifth week postoperatively. The foot was closed primarily. Twenty years later the patient was reviewed. He was a farmer as he had been before the war. His thumb function was restored, the new fingers had some flexion and extension, and sensitivity to touch was present as well as response to temperature. Walking presented no problems[13] (figure 1a-e).

Due to the uncomfortable position of the patient during the period of fixation of the hand to the foot as well as the lack of sensation and motion, this method did not gain wide acceptance. Nevertheless the aforementioned pioneers have led the way to the three basic methods of thumb reconstruction which are still valid today, i.e. osteoplastic reconstruction, digital transposition (pollicization) and toe-to-hand transfer.

The patient with an amputated hand has been and still remains a challenge for a reconstructive surgeon who tries to recreate gripping ability. In 1917 Krukenberg described an imaginative procedure to convert a forearm stump into radial and ulnar rays[14]. The two opposing rays created by this procedure provide a pincerlike grasp that is motored by the pronator teres muscle. Candidates for this operation are the bilateral upper extremity amputee, especially if sight is also impaired. In 1920 Oehlecker transplanted the ipsilateral big toe to the distal end of the radius in a 10-year old boy with an amputated hand at wrist level[15]. However, in the majority of cases either a prosthesis was prescribed or nothing at all.

Although the principle of transposition of tissue on its vascular pedicle had already been described by Monks in 1898[16] and by Esser in 1917[17] (who called it the island flap), it took until after the second World War before it was widely applied in hand surgery.
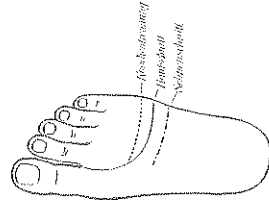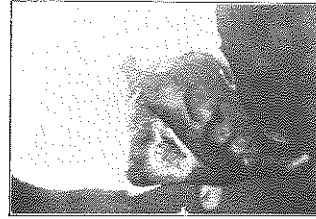
Figure 1a

Figure 1b

Figure 1c

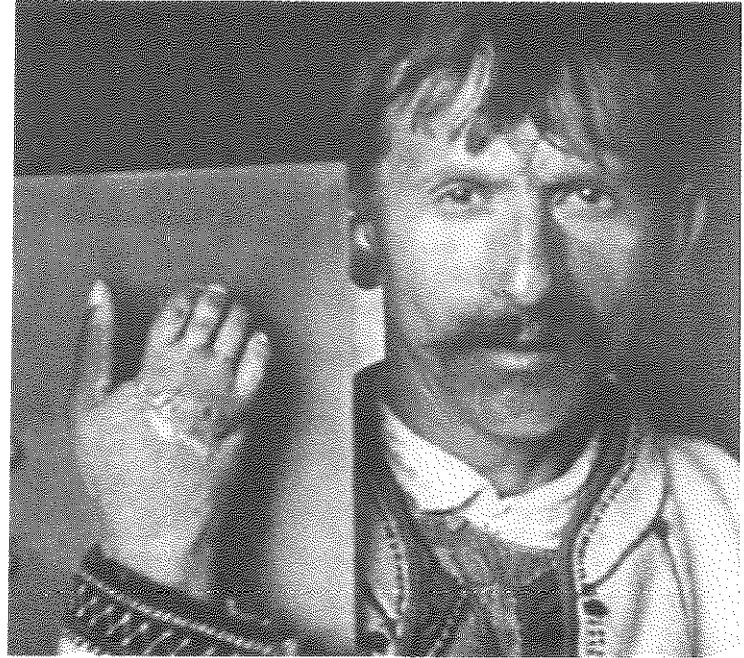Figure 1d

Figure 1e

Figure 1a-e. The transfer of four toes from the right foot to the right hand by J.F.S. Esser in 1916. Preoperative condition (1a, 1b) and hand connected to foot (1b, 1d). Postoperative result of foot-to-hand transfer (1e).

Digital transposition on a neurovascular pedicle was then subsequently described by Bunnell in 1948[18], Gosset in 1949[19], Littler in 1953[20] and Moberg in 1955[21].

The horizon of reconstructive hand surgery was further extended enormously by the introduction of magnifying devices (microscope and loupe) together with the development of micro(neuro)vascular surgery in the sixties.

A.1.3 Microsurgery and Replantation surgery: a retrospective view

Around the turn of the century experiments had already been carried out involving reconnection of transected extremities. In 1897, Halsted[22] transplanted the hind leg from one side to the other leaving the principal artery intact. In 1903, Höpfner[23] replanted three hind limbs in dogs. Although survival rates were short (1, 6 and 11 days) it was clearly the beginning of replantation surgery.

Anastomosing blood vessels in such a way that they would remain patent was obviously the key to further progress in this field of surgery. Von Horoch (1888)[24], Murphy (1897)[25] and Dörfler(1899)[26] all performed end-to-end suturing of vessels. Murphy attributed his low patency rate in healthy vessels to infection. Dörfler applied continuous suturing of all layers of the vessel wall, a detail which proved to be very important. In 1906 Carrel and Guthrie[27-29] used a triangular vascular repair to perform successful auto-replantation and transplantation experiments of the dog leg, scalp and kidney. These surgeons created the basis for modern vascular surgery. Macrovascular surgery became more reliable through this technique, especially in larger vessels with a diameter of more than 4 mm. It is, therefore, surprising that so much time passed, before the first successful replantation of an arm in a human subject using macrovascular techniques was performed by Malt in Boston in 1962[30]. Shortly thereafter, in 1963, Chen in China[31] reported the first successful reconnected forearm, while in Europe Vogt was the first to replant a completely amputated arm in 1966[32,33].

In the beginning of the nineteenth century results of small vessel surgery were very poor. Still in 1948, Shumacker reported a 50% successrate in repairing abdominal vessels of about 3 mm in dogs[34].

To visualize the vessel lumen better and to allow correct placement of vascular sutures for anastomosis of small vessels the need for magnification was recognized. Microscopes had already been introduced for clinical use in middle ear operations in 1921 by the E.N.T.-surgeon Nylen[35]. Ophthalmologists were the second group to use the microscope[36].

It was, however, not until 1960 that microvascular surgery was clinically applied to succesful repair of 2 mm intracranial vessels by Jacobson and Suarez[37,38']. These surgeons

worked with large sized instruments and thick sutures. The need for fine needles, sutures and instruments therefore emerged. Consequently, in the sixties laboratories were set up for experimental microsurgery.

A number of microsurgeons have made a tremendous effort to design and develop instruments and techniques in order to solve the problems in suturing vessels with a diameter of up to 1 mm and less[39-43].

Great pioneers in this field were: Sun Lee, who developed microsurgical techniques in experimental organ transplantation in the early sixties[44]; Buncke, who performed rabbit ear replantations, thumb and index replantations as well as toe-to-thumb transfers in the rhesus monkey from 1963 onwards[45-47]; Goldwyn, who transferred free groin flaps in dogs also in 1963[48]; Krizek[49] and Strauch[50], who reported larger series with long term survival, using experimental groin flaps in 1965 and 1967, respectively and O'Brien[51], who had no failures with 7 groin flaps in the rabbit. It was only the beginning of numerous reports in this field.

The first clinical free vascularized toe-to-thumb transfer was reported in 1966, and carried out by Yang in China. The first clinical free vascularized flap reported was performed by Antia and Buch[53] in 1967, who transferred a free vascularized abdominal dermo-fat graft to the face. Cobbett[54] followed in 1969 with the first free toe-to-thumb transfer in the Western world; McLean and Buncke[55] in 1971 with the first free omental flap to the scalp; Daniel and Taylor[56] in 1973 with a free groin flap and many others followed carrying out free flaps in successive years. The groin flap proved to be the work-horse in the initial years of clinical free tissue transfer. The description of the vascularity of the groin flap and the concept of an axial vessel pattern to nourish the flap provided the essential stimulus for further development in free tissue transfer.

As a result of microanatomic studies all over the world, a "new generation" of free flaps with a large sized, reliable axial vessel pattern has been designed, such as the skin/sub-cutaneous flaps (for example the groin flap[56-58], the scapular flap[59,60] and the parascapular flap[62,63]); the fascial and fasciocutaneous flaps (for instance the radial forearm flap[64-67], the lateral arm flap[68,69] and the dorsalis pedis flap[70-73]); the muscle and musculocutaneous flaps musculocutaneous flaps (such as the lattisimus dorsi flap[74-76], gracilis flap[77,78] and the rectus abdominis flap[79-81]); the osteocutaneous flaps (for example the radial forearm flap with bone[67,82,83], scapular flap with bone[84,85], groin flap with iliac crest[86,87] and the fibula with skin island[88,89]), and last but not least the toe-to-hand transfers with or without dorsalis pedis flap (such as the great toe-to-hand transfer[54,90-92], the second toe-to-hand transfer[52,93,94], the multiple toe-to-hand transfer[95-97], the wrap around procedure[98] and also joint transfers[99,100]).

A number of the aforementioned flaps not only provide adequate coverage, but can also be used as functional tissue. A few examples are the functional muscle flaps, in which the nerve

of the muscle is connected to a motor branch at the recipient site[77,78]; the radial forearm flap with vascularized tendon or nerve[67,101] and the toe-to-thumb transfer[90-97].

With regard to replantation, experiments done at the turn of the century, were taken up again, but this time with the application of microsurgical techniques. Kleinert and Kasdan in the U.S.A. achieved the first revascularization of an ischemic thumb in 1963[102]. In 1965, Komatsu and Tamai in Japan became the first in the world to replant an amputated thumb[103]. In the late sixties and early seventies numerous reports about successful replantations appeared all over the world[104-110].

Survival without adequate reinnervation, however, is of little functional value. The repair of severed peripheral nerves should, therefore, receive special attention. The use of microsurgical techniques in nerve repair[111-115] as well as the knowledge obtained clinically and experimentally considering internal neural anatomy, biology of nerve regeneration and factors influencing nerve repair[116-120] has greatly contributed to the success of replantation surgery (see also A.3.3.1).

A.1.4 Microsurgery and replantation surgery: a current view

Free vascularized tissue transfers play an important role in reconstruction of the upper extremity. It should however only be used if tissue is not available near the recipient site. Especially in trauma, often resulting in large soft tissue defects in combination with exposed bone, nerve, vessel or tendon, free flaps can be very useful not only to cover the wound, but also to restore function[121-124] (see A.1.3).

Toe-to-thumb or toe-to-finger transfers either in post-traumatic or congenital cases have made it possible to reconstruct an amputated thumb or to substitute the loss of multiple fingers[125-129]. Toe-to-thumb transfers can provide a pinch grip and grip strength up to 70% of the normal side, with a two point discrimination ranging from 2 to 15 mm[125,126, 130].

In large centres where routine microvascular surgery is performed overall survival rates from series of 194[131] and 372[132] free flaps are 94% and 91%, respectively (see A.1.5). All centres report a higher failure rate in the initial years of microvascular surgery. Traumatic or post-traumatic cases are less successful than non-traumatic cases (91% compared to 97%)[133].

In a modern, adequately served community replantation surgery is a must. It has become much more than an occasional spectacular surgical event, and is now routine work in most developed countries[123,132]. Replantations should be performed in specialized centres with a round-the-clock service. A team should be available consisting of surgeons, both senior and

junior, anaesthesists, nursing staff, and professionals of rehabilitation, including physiotherapists, occupational therapists and social workers[132,134].

In the ideal situation an experimental microsurgical laboratory is in close relation to the hospital to provide facilities for training and experimental work[123]. Micro- (neuro)vascular surgical expertise is obtained by training and an adequate clinical workload. One clinical case every 3 months does not maintain this expertise and because of this there is little, if any place for the occasional microvascular surgeon. Consequently, to justify an expensive infrastructure of sufficient professional personnel and equipment a replantation centre should drain at least a population of 3 - 4 million people, depending on the degree of industrialization of the area[132].

Replantation operations are long and tiring. They last from a few hours in the guillotine amputation of a digit in a child to 20 hours or more in multiple digit amputations or in hand amputations with extensive avulsion injury of the forearm. It is therefore only under the aforementioned conditions that a replantation centre can deliver a high quality of work over the years.

The decision to replant or to amputate should be made by a surgeon experienced in these techniques after seeing the patient. Many factors are to be considered, such as level of amputation; type of injury - clean laceration, crush or avulsion - handedness, occupation and age; the presence of coexistent disease or trauma and last but not least the patient's wishes. The duration of cold or warm ischemia is extremely important in major amputations; in digits it is important but less critical. Transportation of the amputate in a dry plastic bag surrounded by iced water (about 4°Celsius) is essential. This subject is extensively discussed in paragraph A.2.2.2. The final decision may not be made until an initial exploration has been carried out[134].

The radial aspect of the hand is the site of the majority of the amputations, i.e. the thumb, index and middle finger. The most common level of injury is at the proximal phalangeal level[108,135-138]. The initial operation may be only the first in a planned treatment programme, although as much as possible should be restored right away. Rigid stabilization of the skeleton is usually accomplished by internal fixation. Damaged joints are either repaired, or arthrodesed or silastic prostheses are inserted at metacarpo-phalangeal level. Transected tendons are reapproximated. In replantation surgery normally for every repaired artery two veins are restored. In extensive injury reversed vein grafts at the arterial side and normal vein grafts at the venous side are mandatory. Nerves are usually repaired directly, but a nerve graft may be necessary. Vital skin cover is essential, especially if bone, nerves or vessels are exposed. Skin grafts, local or distant flaps, are required if the wound edges cannot be closed without tension.

In the early postoperative period close monitoring either clinically (colour, capillary refill and needle puncture bleeding) or by device (temperature, transcutaneous oxygen, photoplethysmography, ultra sound doppler or laser doppler flowmeter)[139] can detect vascular compromise. This may require various measures such as positional adjustment, release of constrictive dressings or exploration with reanastomozing of thrombosed vessels or interpositioning of vein grafts.

Following replantation or revascularization specifically trained personnel should be brought in, including physiotherapists, occupational therapists, and technicians with facilities for training and splint manufacture[140-142].

Success rates of several large series range from 57%-93%[136,138,143,144] (see A.1.5). These numbers, however, only indicate survival and not functional recovery. Assessment of functional recovery should not be compared with a normal limb, but with the alternative, namely amputation. In digital replantation surgery, thumb replantation gives the best functional result and should be attempted at every opportunity[132,134,145]. If all fingers are lost or severely injured, some or all of them should be replaced to provide a pinch grip. Single finger replantation is less indicated, especially if the level of amputation is proximal to the proximal interphalangeal joint[146]. However, for instance in the young child or in a musician, single digit replantation, if feasible, is usually carried out. The more distal the replanted part the better the prognosis[147]. A partly amputated finger should always be revascularized, although the indication can be discussed if only the index finger is involved[148]. Hand and arm replantation has proved its value[138]. Especially in a sharp injury just proximal to the wrist[143]. In limb replantation most patients have accepted the aesthetic and functional result, and although usually shorter with diminished power and sensation, it is considered superior to what a prosthesis could offer. In the major replants, generally intrinsic muscle paralysis results, which necessitates multiple tendon transfers at a later stage.

Chen et al published in 1978[138] a follow-up of 214 patients for more than 1 year and assessed them in four ways: Grade 1: 73 patients (34.1%) were able to resume their original work, had a range of joint motion that exceeded 60% of normal, had complete or nearly complete recovery of sensation and had muscular power of grades 4 - 5. Grade 2: 72 patients (33.6%) were able to resume some suitable work, had a range of joint motion that exceeded 40% of normal, had nearly complete recovery of sensation of tissue supplied by the main sensory nerves and had muscular power of grades 3 - 4. Grade 3: 60 patients (28%) were able to carry on daily life, had a range of joint motion that exceeded 30% of normal, had partial recovery of sensation and had muscular power of grade 3. Grade 4: 9 patients (3.9%) had almost no functional recovery although the replanted limb survived.

When compared with other centres with the same grading system these figures show only

slight alterations with a lower percentage in grade 3 and a higher percentage in grade 4[136, 143, 149].

Late complications of replantation of digits include intolerance to cold, stiffness and anaesthesia. Cold intolerance almost always occurs, however, it does subside in the following years. Stiffness is related to level of injury, success of secondary tendon and joint surgery, and cooperation of the patient in the rehabilitation process. Sensory recovery can be very good if the replants were distal, the injury sharp, the patient young and sensory reeducation given. The sensory recovery is poor in crush or avulsion injuries, in the older patient and when sensory reeducation has not been prescribed[150]. In a reported series of 74 replanted digits the order of sensory recovery was pressure, touch, pain, cold, warmth, and perspiration with a mean time of first appearance varying from 10 to 27 weeks[151]. In summary the best result is achieved in the young patient, with a sharp or localized crush injury, who is entered early into the rehabilitation process and is motivated to cooperate.

A cost-benefit analysis of replantation surgery is described in paragraph A.1.5.

A.1.5 Microsurgery and replantation surgery in Rotterdam

Experimental microsurgery and small vessel surgery started in Rotterdam in the 1970's, in the Laboratory for Experimental Surgery[152]. Stimulated by the department of general surgery and later also by the department of plastic and reconstructive surgery, W.J. Kort has developed a separate laboratory for experimental microsurgery. Apart from the many research projects which have been completed in this lab, also a microsurgical course is organized twice a year, since 1981. Furthermore all residents in plastic surgery receive their basic microsurgical training in this laboratory.

In 1976, at our department the first clinical replantation of a guillotine amputated digit was performed by G.A.M. Malfeyt and J.M. Vaandrager. The first hand was replanted in Rotterdam by K.E. Bos in 1977. K.E. Bos can be seen as the founder of clinical microvascular surgery in Rotterdam[130,153]. The first free vascularized flap in the Netherlands was a groin flap performed by him in 1976 in Rotterdam, together with W. Boeckx from Leuven, Belgium. His successor A.R. Smith performed microsurgery on a more regular basis. He organized a replantation team in 1980[154]. Experimental work was also initiated in this period, concerning preservation of ischemic tissues[155] as a preliminary to composite tissue transplants as well as clinical postoperative monitoring of microvascular surgery[156] (see A.2.2.2).

The author and co-workers have been performing microvascular surgery since November

Replantations / revascularizations

250

214
200
200

139

150

108

100

46

50

30

0

1976–1980   1981–1985   1986–1990

Free vascularized tissue transfers

140

122

120

111

100

80

60

52

47

40

23

19

20

0

1976–1980   1981–1985   1986–1990
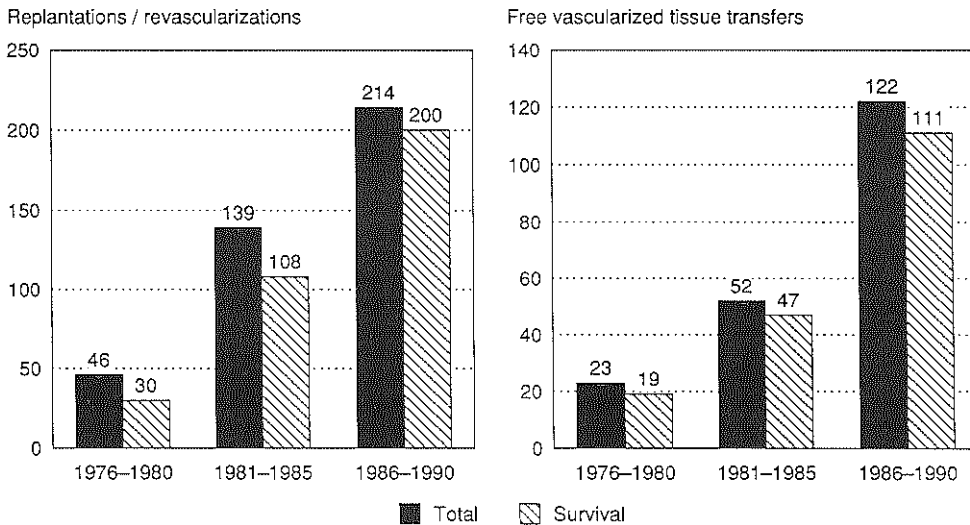
■ Total   ▨ Survival

Table 1 (left) In the last 15 years a total number of 339 parts were replanted or revascularized in 272 patients. In the last five years 54% of the total amount was operated. The survival rate of the last group was 93%. Attempts to replant were not included. **(right)** In the last 15 years a total number of 197 free vascularized flaps were performed. In the last five years 63% of the total amount was operated. The survival rate in this period was 91% .

1985 on a routine basis. 63 % of the flaps and 54% of the replantations and revascularizations listed in table 1 were operated in the last five years. Survival rates during this period were 91% and 93% respectively (figure 2a-d). The laser doppler flowmeter was introduced in 1985 for postoperative monitoring of microvascular surgery and in October 1990 4 instruments were installed in a specific nursing unit in our department. The data from this monitoring system are collated by an especially designed computer programme. The laser doppler flowmeter has facilitated greatly the decision to reexplore following vascular compromise, resulting in better survival figures (table 1)[157,158] .

This thesis is an example of our experimental work in the last five years.

Replant surgery is costly for health insurance organizations. The expenses, however, are much higher if replantation is not carried out and the patient does not have a change to return to work. A retrospective analysis in 1983 of 34 patients in whom replantation was carried out, 21 were successful, saving 6.5 million guilders, provided all patients reach the age of 65 years. From a macro-economic view, therefore, the benefit for the total community is clear[159].

Microsurgery is here to stay!
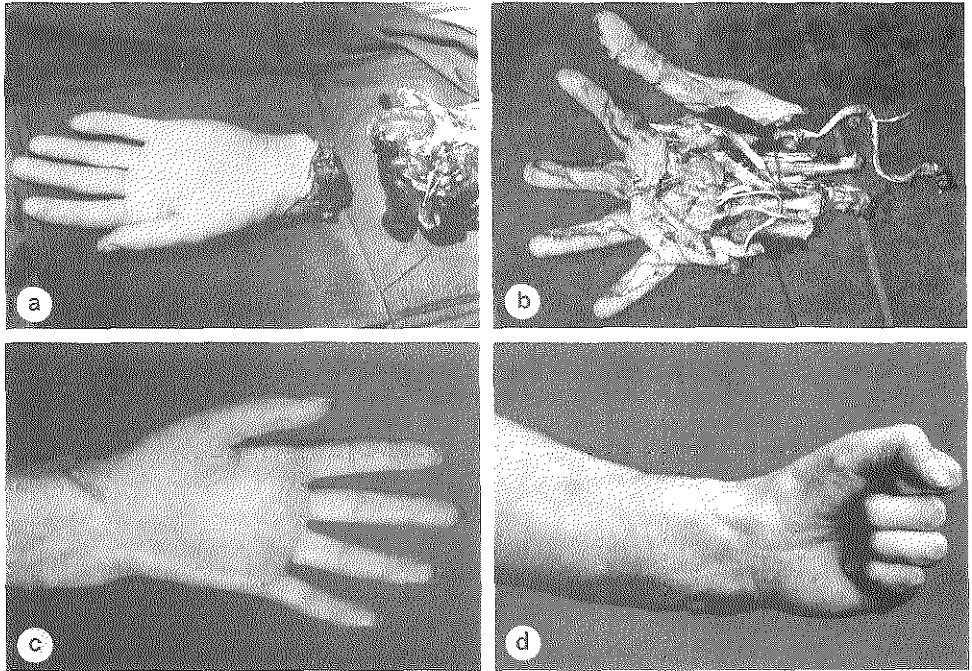
Figure 2 a) Hand amputation with moderate crush: replantable; **b)** Hand amputation with severe crush: non-replantable; **c)** Following replantation (same patient as 'a)'), full extension is possible; **d)** Following replantation (same patient as 'a)'), full flexion is possible.

A.1.6 Hand injuries

Research has been carried out in our department to calculate the number and incidence of hand injuries in 1982 in the Netherlands (15 million inhabitants). In that particular year the number of hand injuries was 70,000, of which 30,000 were industrial injuries. About 10% of these injuries (generally without replantation), resulted in permanent invalidity. The average absenteeism from work was 33 days. The majority of patients were 20 to 35 years old. In 1982 it has been calculated that direct costs for medical care of hand injuries were about 120 million guilders, while indirect costs such as benefits were about 363 million guilders[160].

The Dutch "Privé Ongevallen Registratie Systeem PORS, Stichting Consument en Veiligheid", registers injuries, with exclusions of traffic and industrial injuries. They report the number of injuries to the upper extremity as 280.000 per year. The percentage of injuries of hand and fingers in this report in relation to the rest of the upper extremity averages 58% (figures of 1986 - 1989)[161].

In the U.S.A. (250 million inhabitants), the National Safety Council's Accident facts[162], reported that the number of disabling work injuries in 1986 was 1,800,000. Of this total, 160,000 were arm injuries, 90,000 hand injuries and 250,000 finger injuries. The National Institute for Occupational Safety and Health in collaboration with the Consumer Product Safety Commission reported that in 1982 a total number of 1,129,392 occupational hand and finger injuries were treated in hospital emergency rooms (35% of total injuries)[163]. Total arm, hand and finger injuries constituted 20% of the compensation costs of all injuries. Costs of work accidents in 1980 were 30 billion dollars, all costs of accidents were calculated at 83 billion dollars, again 20% of these costs involved the upper extremity. In their extensive survey of the frequency and costs of upper extremity disorders in the U.S.A., Kelsey et al[164] postulated that indirect costs of lost earnings and compensation was more than double the costs of actually treating these injuries.

Burke's study in Great Britain suggests that 475 new hand trauma cases requiring hand surgery expertise occur in every 100.000 population each year. The mean age of patients was 30 years, most were males. Nearly 18% of all injuries involved the hand and wrist. He states that the enormous indirect costs of hand disorders, due to loss of employment and compensation, could be greatly reduced if co-ordinated services for care of the hand could be developed throughout the country[165].

In the U.S.A. about 20.000 amputations occur each year[166]. Nylander et al, from Sweden reported in 1984 an incidence of 110 serious amputation injuries per year (8 million inhabitants). Criteria for serious amputations were amputation of the thumb proximal to the IP joint, two or more fingers amputated through or proximal to the PIP joint and hand or arm amputations. In their study in 70% of the cases, replantation would have been technically possible but has been only carried out in 10%[167].

All these numbers are difficult to obtain and interpret. For instance the number of amputations of the hand in the Netherlands in the years 1987 - 1989 were supposed to be 22, of which 13 were replanted[168], these figures do not agree with our own figures. A more extensive coding system is necessary to provide exact numbers and percentages.

A.2 The hand amputee

The patient without a hand (the hand amputee) is the ultimate challenge for the reconstructive hand surgeon who has to recreate gripping ability. Historical remarks on conventional methods of reconstruction of hand amputations are described in A.1.2.

A.2.1 Reconstruction of the hand amputee

The first microvascular reconstruction of the antebrachial stump by transplantation of two separate toes in order to create a grip, occurred in China in 1979[169]. The stump was lengthened on this occassion with a metallic implant. The toes were positioned on the implant opposing each other. Following many complications, they changed their method using two separate toes with long metatarsals, which were placed at the end of the stump opposite each other[170]. These procedures are very demanding and have gained little popularity.

In 1985, Vilkki[171] published a method of reconstructing gripping ability in hand amputees. He accepts the length of the stump and uses it as a counterpoint or post for the grip. The transplant consisted of a second toe with its metatarsal bone and a hemipulp flap from the lateral side of the big toe. The main indications were victims of hand amputation who are also blind. Bilaterally injured patients and in some unilateral hand amputees this method can also be used. Pinching power in six patients were 5.5 - 11 kg with an opening range of 3 to 4.5 cm. The limitations of the new grip and the subnormal sensibility can be a disadvantage, although patient acceptance is better than with a Krukenberg procedure[14,172] (see A.1.2). The aforementioned methods do not preclude the use of functional or aesthetic prostheses.

A.2.2 Alternatives to reconstruction

Alternatives to current surgical reconstruction of the amputated hand can either be nothing at all, a prosthesis or the prospect of allogeneic hand transplantation.

A.2.2.1 Hand prostheses

> *"Though engineers and prosthetists have made substantial contributions, they need perspective and humility to inspire and guide the very long, sustained efforts required to replace even a few of the roles of the hand."* [E.F. Murphy, 1970[173]]

The acquired or congenital absence of any portion of the hand and arm results in a visible and functional disability. The patient or parents of the affected child have to accept the loss or absence of the upper extremity or a part of it, otherwise it is unlikely that a successful

Figure 3 a) Following non-replantatble forearm amputation, the elbowjoint was saved using a free vascularized m. latissimus dorsi flap with split skin coverage. b) The same patient with a myoelectical prosthesis manufactured by O. Bock, Son en Breugel, The Netherlands.
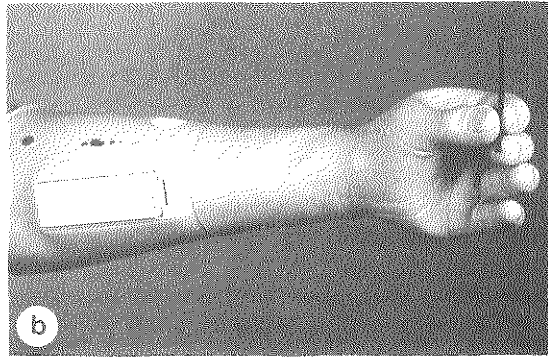
rehabilitation and functional use of a prosthesis will be achieved, if a prosthesis is considered. The acceptance must become the primary focus in the total rehabilitation process[174].

The human hand is an extremely complex device with very delicate sensory and motor function capabilities. Even the most advanced hand and hook prosthetic devices, cannot provide the functional restoration to individuals with complete hand deficiencies[175].

In traumatic upper limb amputations it is essential to understand the needs and expected capabilities of the patient before application of a functional prosthesis. An immediate or early fitting of a prosthesis can be beneficial, however, if not properly managed the results can be detrimental[176,177]. In congenital limb deficiencies a passive hand prosthesis can already be introduced at the age of 3 months. The passive hand will provide gross opposition during the early stages of eye-hand coordination as well as the development of the child's tolerance to a prosthesis.

As gross palmar prehension and hand dominance develop at approximately 8 months of age[178], the introduction of a functional prosthesis should be consistent with this. Upper limb prosthetic terminal devices are designed as mechanical or electromechanical systems and are either biomechanically or electrically powered or a combination of various systems.

Before the clinical use of electric hands in the sixties, first developed in 1948[179], considerable controversy existed with respect to the use of mechanical hands or hooks. The hand being more aesthetic, but heavy and awkward as compared to the more functional hook.
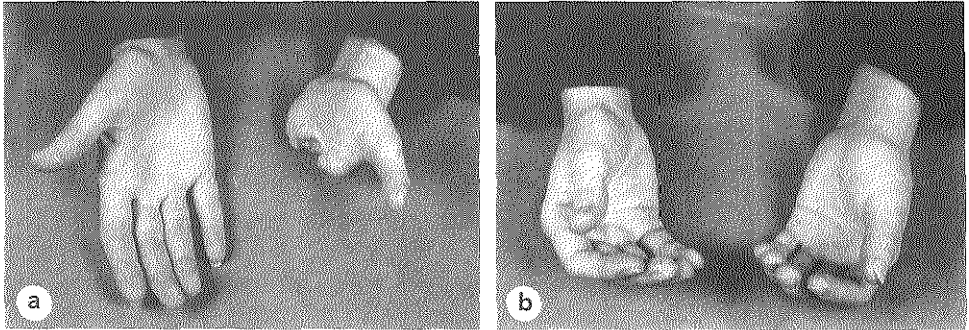
Figure 4 a) A patient with a congenital hand malformation. b) The same patient with an aesthetic prosthesis manufactured by J. Pillet, Centre de Prosthese Plastique, Paris, France.

Conventional mechanical prostheses, however, are reported to be lying in the cupboard in more than 50% of the individuals provided with them[180]. The introduction of the prosthetic hook early in the rehabilitation process is possibly the major factor in rejecting a prosthesis, because it interferes with the psychological needs of the particular individual at that time[181].

Biomechanically powered devices function through body movement[182,183], electrical devices from a battery. They are the source of power for various control systems of the device, such as cable control, myoelectric control and switch control. The prosthetic hand serves as an assistent to the sound hand, just as the non-dominant normal hand is related to the dominant normal hand. The device is important for gross grasp, such as holding and stabilizing objects, while the normal hand performs the fine motor prehension activities.

The electric powered prosthetic hand device, which is controlled using electromyographic (EMG) potentials from opposing muscle groups within the residual limb, will best serve the need to perform most functional tasks during daily life. This is especially valid for the patient with unilateral upper limb involvement (figure 3a+b)[184-186].

The aesthetic prosthesis fulfills the need of the person with an upper limb deficiency, i.e. the wish to go unnoticed and have two hands like everybody else. An example of an aesthetic prosthesis with an additive passive function in a congenital amputee is demonstrated in figure 4a+b[187].

A prosthesis, however, can never replace any functional restoration of hand function which can be achieved following a traumatic or congenital limb deficiency.

A.2.2.2 Allogeneic transplantation of the human hand

> *There is no place for the "bullseye" psychology, "Let's put*
> *it in, give the drug, and hope we hit the mark. "*
> [J.E. Murray, Editorial P.R.S. 1964[188]]

In 1944, Hall[189] published a detailed protocol for an operative procedure for human homologous upper extremity transplantation. He presented a human cadaver transplantation of the arm, performed at mid-humeral level. Hall also described the problem of infection, vessel anastomosis and thrombosis, osteosynthesis, preservation and the necessity of the experienced surgical team working in a well equipped hospital. He did not mention however, the role of nerve regeneration nor the role of rejection.

An allogeneic human hand transplantation was carried out in 1964. It was transplanted from a fresh cadaver and remained viable about 14 days. The patient had received cortisone and azathioprine as immunosuppressive agents. Local irradiation was also applied to the graft[190].

If an allogeneic human hand transplantation could be performed today the following factors should be considered, i.e. technique, preservation, immunology (rejection), functional recovery, rehabilitation and logistics concerning transplantation.

The technique of the actual transplantation can be compared to a replantation procedure at the same level routinely used in large replantation centres (see A.1.4).

With respect to preservation of the larger donor graft a few remarks on the effect of tissue anoxia of the transplant should be made. In an ischaemic situation tissue reacts with the formation of oedema, increasing potassium levels and metabolic acidosis due to a rise in lactate. These factors increase with prolonged ischemia and are inhibited by cooling. The cooling effect is best in iced water (4° Celsius).

Usui et al[191] demonstrated in an experimental study on canine limb replants that eight out of ten dogs could tolerate 12 hours of ischaemia before revascularization following cooling by iced water as opposed by only eight out of 15 survivals when the amputated limb was kept at room temperature for six hours. Cooling had no effect on the lactate level which actually increased. The change in lactate and pH were found to cause marked differences between the dead and surviving animals.

Muscle tissue is particularly sensitive to ischemia, histologic changes can be detected following two hours of ischemia, whilst rapidly increasing irreversible damage will occur following six hours of ischemia at room temperature. Recovery of nerve tissue conduction following ischemia takes a couple of minutes after two hours, and about 30 minutes after six

hours. Following eight hours, however, intra fascicular oedema occurs and nerve function does not recover[192,193].

Fingers can tolerate anoxia up to 8-10 hours at these temperatures as they do not contain muscle tissue. Fingers cooled in iced water have been reported to be successfully replanted following 39 hours of ischemia[194,195].

The maximum hypothermic ischemia in replants containing muscular tissue has been investigated by van Alphen et al[196]. He replanted the latissimus dorsi muscle following 5, 8, 16 and 24 hours of cold ischemia. In the first three groups slight to severe degenerative changes, with a severe reactive proliferation of fibroblasts were seen. Degenerative changes were seen more often as ischemia time lasted longer. In all sections of the 24-hour group total or nearly total necrosis was found. Cold ischemia alone therefore, will not be sufficient enough to prevent muscle damage if a planned hand transplant has to be carried out with a donor graft, when there has been a long interval between disconnection at the donor site and revascularization at the recipient site.

Organ preservation has concentrated since the late sixties on cold storage in special solutions, as described by Collins[197] as well as machine perfusion, as described by Belzer[198]. These methods have remained essentially unchanged for the past 20 years. They were effective for kidneys but not so effective for livers, pancreas and hearts. Until 1987, therefore, these organs were transplanted as emergency procedures with safe preservation times of about six to ten hours. The mechanisms of preservation-induced organ damage are not yet fully understood[199,200]. Preliminary clinical results at the University of Wisconsin (UW) have shown successful transplantation of pancreas and liver after about 20-24 hours of cold storage preservation using their UW-solution. This is in accordance with other reports[201] achieving extended preservation with UW solution as compared to Euro-Collins solution. The need for emergency procedures in these organs has therefore been eliminated[202]. Machine perfusion with special solutions is still experimentally investigated. For instance Ploeg et al[203] obtained 63% survival following five day perfusion preservation of the dog kidney.

Another solution is Fluosol DA-20%[204] (an isotonic 20%-solution of Fluorocarbons), which has a high affinity for oxygen and hence can act as a tissue oxygenator. In a study at our Institution in five patients with traumatic amputations, the amputated part was perfused, with a pulsatile pump at a mean arterial pressure of about 90mm Hg, using Fluosol DA-20% for between 16 and 46 hours. Following eight hours of perfusion a 10% increase in weight occurred, which gives an idea of the degree of interstitial oedema. Parameters such as oxygen tension, potassium, lactate, glucose and adenosine-triphosphate suggested that the tissue was sufficiently provided with oxygen and viable after many hours of perfusion. One

perfused digit was even successfully replanted following 17 hours of perfusion and 24 hours of ischemia[205]. Unfortunately the American Food and Drug administration did not approve the solution, therefore, further research with Fluosol has stopped. It is beyond the scope of this thesis to discuss all different kinds of storage or perfusion methods and solutions for preservation, although the need for good preservation in case of hand transplantation is clear.

The importance of immunology and rejection are obvious and are described in chapter I, section B.

With regard to functional recovery the results of reinnervation should be compared with the results of replantation following guillotine amputation at the same level (see A.1.4). Questions which have to be resolved concerning this subject are the influence of immunosuppressive agents and the influence of rejection on nerve regeneration and ultimately functional recovery.

Cyclosporine A (CyA) is currently the most used immunosuppressive drug in transplantation surgery. Amongst other adverse effects it can also be neurotoxic[206,207]. Most presentations of neurotoxicity in humans involve the central nervous system with seizures, cerebellar disorders, neuropathies and other problems[208]. Tremor and minor sensory loss, particularly of the extremities, are stated to be the most common symptoms of neurotoxicity and often disappear with continued treatment. On the other hand, prolonged high levels of CyA are known to increase side effects in general[209].

It has been noted that CyA can significantly improve functional testing in the early postoperative period in syngeneic nerve grafts in rats as compared to nerve grafts without CyA treatment[210]. CyA may promote nerve regeneration across the nerve anastomosis, by inhibiting wound healing and fibroblast migration, and it may also promote revascularization of the nerve graft[211]. Methylprednisolone, being the other suppressive drug in our protocol is not known to cause neurotoxic effects, unless it is administered intrafascicularly in the nerve[212].

Samulack et al[213] reported that under immunosuppressive therapy in normal glabrous skin in baboons latency times of single axons are prolonged. These data suggest a neurotoxic effect. In allogeneic non-rejected skin of composite tissue transplantation under high levels of CyA, in the same study, latency times of single axons were also prolonged compared to normal glabrous skin. The most significant differences in latency times of single axons, however, were encountered in rejected allogeneic glabrous skin.

With respect to the role of rejection on nerve regeneration, there is a definate negative influence[213].

Rehabilitation following hand transplantation is essential, but will be technically the same as in a replanted hand (see A.1.4).

Logistic problems concerning the acquisition of a donor hand should be solved by public information, changing of the existing general donor codicil[214,215] and linkage with the Eurotransplant organization[216].

This thesis aims to make a contribution in the long struggle before actual human hand transplantation can be performed safely.

A.3 The research model for experimental allogeneic transplantation of the hand

The history of experimental allogeneic limb transplantation, including two hand transplantation studies in baboons, is described in Chapter I.B.1. From an anatomical, immunological and functional point of view, studies in the higher developed non-human primate can be better extrapolated to the human being than studies in rodents and dogs. In the major part of the introductory studies and in the main project, therefore, the rhesus monkey was chosen as the experimental animal (see Chapter I.C).

A.3.1 The rhesus monkey

The hands of the various primates, including man, are very similar, their minor variations are adaptations to their special activities. Only the primates have opposable thumbs. Opposition is a compound movement of abduction, flexion and medial rotation occurring at the carpo-metacarpal joint of the thumb. The thumb is either absent, rudimentary, or is like the other digits in other mammals. In the later developed monkeys and apes, the thumb is smallest and least opposable in arboreal forms. Monkeys that dwell on the ground have much better developed thumbs. In man, the thumb shows the greatest specialization and development in strength, opposition and size. Next in order come the great apes, the gorilla, baboon and chimpanzee[217]. The rhesus monkey lives in the trees as well as on the ground. The thumb is reasonably well developed. The pollical index, or percentage of length of the thumb to that of the long finger, is comparable to that of the gorilla and chimpanzee[218]. The rhesus monkey belongs to the Old World monkeys. They possess a saddle joint at carpo-metacarpal level which makes opposition possible[219]. The flexor pollicis longus muscle, however, is less developed in the rhesus monkey than in man, the extensor pollicis brevis muscle is absent and the thenar muscles although present are less developed. These factors make opposition less refined. The arm and hand nerves throughout primates are practically the same. Detailed descriptions on anatomy and function with regard to our research model

are provided in Chapter II.B.

A.3.2 The set-up for operation

All experimental studies on rhesus monkeys were performed at the "Primate Center" in Rijswijk, The Netherlands.

At this centre four monkey cages in an isolated temperature controlled room were at our disposal. A veterinary surgeon and two veterinary assistents helped us to take care of the animals before, during and after operation. The operating room consisted of two operating tables with all necessary equipment for modern surgery, including two artificial ventilators. An operating microscope (Contraves-Floorstand OPMI-MDM) was provided by Zeiss. We used basic fine surgical instruments, with addition of a microsurgical set. At the day of operation two anesthesists with an assistant, an operating nurse, a veterinary assistant (with operating experience) and both authors were present (see figure 5).

The operative procedure is extensively described in Chapter III.B.2.2.2 and III.A.2.4.

A.3.3 The set-up for functional testing

As has been stated before, functional recovery is essential following hand transplantation. Consequently, the design of the research model had to be a complete functional unit, with separate recovery of sensory and motor function. Therefore, our research model, included amongst other nerves, the median nerve with the motor branch to the thenar muscles, as well as the sensory branch with the radial and the ulnar digital nerve to the thumb (Chapter II.B, III.A and C). First some general remarks on nerve injury and repair will be made. Subsequently the set-up for functional testing will be described with a justification of the selected tests in our research model in the rhesus monkey.
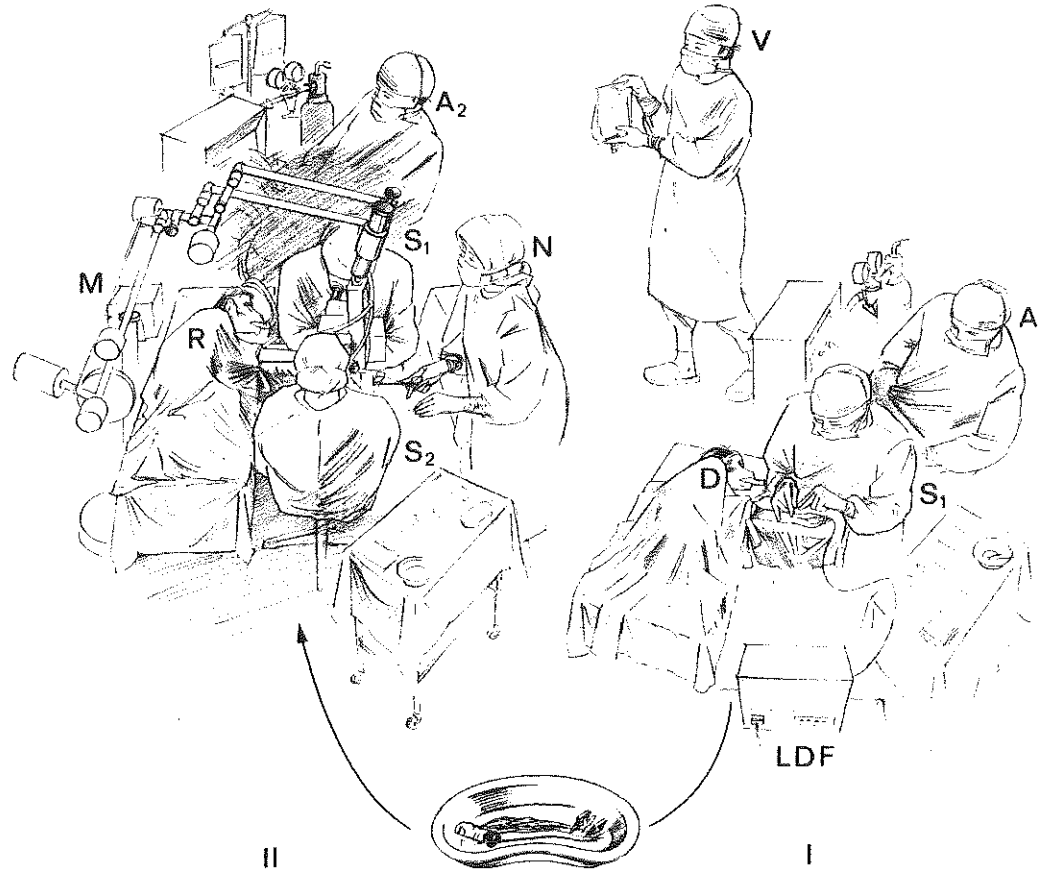
Figure 5. Operating room setup at the Primate Center, TNO, Rijswijk, The Netherlands; D: Donor monkey; R: Recipient monkey; LDF: Laser Doppler Flowmeter; N: Nurse; V: Veterinary assistant; A1: Anaesthesist; A2: Anaesthesist; S1: Surgeon; S2: Research fellow; M: Microscope.

A.3.3.1 Nerve injury and repair

> *"If the central cell body were the height of an average*
> *man, its axon would be one or two inches in width and*
> *would extend more than two miles."*
> [T.B. Ducker, 1980[220]]

The division of a peripheral nerve causes a variety of changes in the nerve cell body, in the proximal segment of the nerve, at the site of the nerve injury, in the distal segment of the nerve, and in the distal endings of both sensory receptors as well as the motor end-plates[119].

The nerve cell will respond with metabolic activity to restore the cytoskeleton of the damaged axon within a few hours of the injury. There is an increase in ribonucleic acid, protein metabolism and lipid synthesis and a decrease in neurotransmitter production. The proximal nerve end degenerates. Depending on the severity of the injury degeneration will occur over a longer distance along the axon. This process can involve one or more nodes of Ranvier (the non-myelinated part of the axon) and even result in death of the cell body[221,222].

At the site of the lesion multiple axons will sprout into regenerating units during the first 24 hours following injury. While first unmyelinated, these units become myelinated in time. At the end of each axon a growth cone with filopodia[223,224] tries to make contact with the distal segment. Probably dependent on the success of this contact the large number of fibres decreases, over a period of months to return to normal[119]. The sprouts which do not make a connection atrophy and disappear or become surrounded by fibrous tissue forming a neuroma.

The distal nerve segment also degenerates following transection (Wallerian degeneration[225]) a process which lasts several weeks and occurs concurrently with axonal sprouting. Schwann cells (cells wrapped around the axon to form a multilaminated myelin sheath) proliferate, the myelin breaks down and is phagocytosed by these cells. Phagocytosis of all cellular debris may take one to three months. Endoneurial tubes (basal lamina of the Schwann cell) of the distal segment collapse, due to the phagocytosis of myelin and axonal components, to form the "bands of Büngner". These bands represent the non-reinnervated endoneural tubes. The proliferating Schwann cells organize themselves into columns. The axon sprouts from the proximal nerve segment are associated with these columns and regenerate between the basal lamina of the Schwann cells, which form new endoneural tubes.

After a few months there is a change in the organisation of firstly the proximal stump and later the gap between the stumps as the axons advance. This process is called compartmentation. There is a reorganisation of the nerve trunk into many small

compartments, each surrounded by a new perineurium.

Compartmentation is more prominent when the nerves are exposed to foreign environment, i.e. in the periphery of a nerve, where the perineurium is damaged. This is probably an effort to reorganise the abnormal endoneural environment, restoring the perineural barrier. After a year there are few or no signs of it left. The clinical implication may be that it results in a change of fascicular pattern, thus making matching of the cut ends more difficult if the repair is done late[118].

The final functional result will depend on the number of axon sprouts associating with Schwann cell columns and reinnervating matching end organs.

Muscle fibres usually atrophy several weeks following transection of their motor nerve. The atrophic muscle fibres appear histologically as round structures with a nucleus, which has migrated to a central position. In normal muscle a random mixture of type I and II fibres can be seen. In muscle which becomes reinnervated clear groupings of one type of muscle fibres can be detected, instead of a random mixed pattern.

The synaptic folds are present for more than 1 year following denervation. Acetylcholine receptors change their position from central to peripheral along the entire muscle, creating a supersensitivity to acetylcholine[226]. As reinnervation occurs, sprouting axons reach the original motor end plates to reform the neuromuscular junctions. These axons also sprout collaterally to reinnervate several muscle fibres, thus creating groupings of muscle fibers of one type, instead of the normal mixed pattern. Sprouting and synapse formation are followed by a process of synapse regression in which redundant reinnervation may be modified in favour of a predominant endplate connection. Furthermore, trophic factors are released by the muscle to influence nerve regeneration. This can be illustrated by the fact that the axon of a sensory nerve placed in a muscle will sprout into the denervated muscle and not into the innervated muscle[227,228].

Sensory receptors (see A.3.3.2) progressively change following denervation. The non-nervous components, however, survive for a long period of time. Recovery of protective sensibility is possible even years after nerve injury. Although the critical time period with regard to the possibility of reinnervation of sensory receptors has not been determined, functional sensation appears to decrease with a delay in repair longer than 6 months following injury[229,230]. The great plasticity of sensory reinnervation is demonstrated by the return of some degrees of pain, touch and temperature sensation in distant non-innervated skin grafts and skin flaps[231-234]. In a reinnervated vascularized tissue transfer, such as a toe-to-thumb transfer, sensory reinnervation assessed by moving two point discrimination can even be better than it was when the toe was still on the foot[150]. The quality and quantity of recovery following the same kind of nerve repair varies between individuals.

Nerve injury causes a breakdown of the blood nerve barrier (in the internal perineurial layers and in the endothelial cells of the endoneurial microvessels)[235] at the site of injury resulting in loss of the normal homeostasis. Following breakdown an immunologic response occurs at the nerve ends inhibiting nerve repair. CyA has been demonstrated to be beneficial in nerve regeneration probably due to modification of the immune response (see A.2.2.2)[211]. Furthermore in the normal nerve there is fast and slow axoplasmatic transport[236]. Material necessary for the structural skeleton moves at a slow rate to the periphery (1-6 mm/day), while material that plays a functional role, for example neurotransmitters move rapidly (~410 mm/day)[237]. Recycled neurotransmitter vesicles are also transported in a retrograde direction serving as a mechanism in controlling the nerve cell body[238]. Following nerve injury the composition of the transported material alters in favour of material necessary to rebuild the axonal cytoskeleton[239].

It is most likely that axonal transport is essential for the trophic effects of the nerve on the target end organs.

Peripheral nerves will regenerate across small gaps with target specificity[240]. For instance discrimination between tendon and nerve[119,241], as well as differentiation between sensory and motor segments[242]. This mechanism of target specificity is called neurotropism and is demonstrated across a gap of 5 mm[243,244]. Along a longitudinal fibrin matrix within a nerve conduit axons elongate much more effectively than through a randomly oriented fibrin matrix or an empty chamber, suggesting the existence of contact guidance[119].

The role of ion shifts near the nerve ends, growth factors, hormones and many other regulators still need to be established[245-250].

The ideal nerve repair should integrate all the aforementioned factors[119]. Clinically established factors in nerve repair which influence nerve regeneration are type of trauma[251], length of the gap[252,253], presence of blood clots and debris in the environment enhancing fibrous scarring[118,119]. Malalignment and stress or tension at the suture line(s) as well as type of nerve repair also play a role. Examples of nerve repair are epineural suturing, perineural suturing, nerve grafting and fibrin glue approximation of nerve ends or tubilisation by various methods[246,254-261]. In our model epineural suturing was performed to approximate the median nerve ends without tension using the microscope. As the nerve is small, epineural sutures were considered to be less harmful than group fascicular suturing. Size was not the only factor to use epineural repair, as there is still controversy regarding the best microsurgical repair method[260]. Extensive research has been and still is performed regarding peripheral nerve regeneration.

Much less, however, is known regarding changes in the cerebral cortex following peripheral nerve injury. In the cerebral cortex, two specialized areas are present, i.e. the

primary motor area (MI) and the primary somatosensory area (SI). These areas are somatotopically organized: each part of the body is represented in the cortex such that adjacent body parts have adjacent representations in any one of the functional areas. Denervation of such specialized cortical areas due to peripheral nerve trauma leaves these parts, though itself anatomically intact, without its functional substrate. In recent years the functional plasticity of these cortical areas has been investigated both immediately after peripheral nerve transection and after subsequent functional recovery of the nerve.

Merzenich et al[262] and Dykes[263] showed that neurons in monkey SI hand area fell silent immediately after deafferentation by median nerve transection. Within hours however neurons in this area could be activated by stimulation of adjacent, non-denervated skin. Within a few months the previously denervated area was almost totally reorganized and filled with response areas from other parts of the hand. When the median nerve was repaired, sensory function in the hand recovered only partly. Neurons in the previously deafferented section of SI could again be driven by sensory input from the matching part of the hand. However, some abnormality in the organization of spatial information persisted.

These comparatively quick changes in cortical representation patterns following peripheral nerve transection suggest that synaptic relations between cortical areas and peripheral sense organs or somatic musculature can be continually reshaped in adult mammals. The persisting abnormalities in cortical somatotopy and the poor sensory spatial resolution that remain after recovery of function following peripheral nerve repair can possibly be due to imperfect matching of fiber bundles between the proximal and distal stump.

A.3.3.2 *Functional testing*

In rhesus monkeys evident limitations exist with regard to functional testing. In our experimental set-up we selected methods, which were not too complicated to perform, allowing frequent testing of function recovery and preferably in conscious monkeys.

Information on longitudinally assessed recovery of sensory and motor function was our main interest. Specific tests for evaluation of sensory receptors (mechano-receptors, thermo-receptors and polymodal nociceptors)[264-266] were not considered as these tests can be performed only as terminal experiments, under general anaesthesia, with exposed nerves combined with single unit electrophysiological measurements[226]. Consequently, for sensory recovery evaluation a small electric current was used in the conscious monkey that

stimulated sensory nerve ends and provoked a withdrawal reflex in case of reinnervation (chapter II.B and III.C).

To evaluate motor function, two basic methods were used. The first method is electromyography[267] and the second method is observation of functional tasks monitored by video recording. Applying the same philosophy as mentioned with regard to sensory evaluation, transcutaneous assessment of compound motor action potentials of the thenar muscles was used to determine muscle reinnervation.

Functional assessment in terms of range of motion of the grafted thumb is hardly possible and very variable in non-operated thumbs[219]. Therefore, video recordings were used to demonstrate functional use of the thumb. If the rhesus monkey repeatedly picked up food morsels with thumb and index finger of the operated side, while the other hand was bandaged, the functional task was scored as positive (see chapter II.B and III.C).

A.4 References

1. Shaw WW: Microsurgery - looking ahead. Clin Plast Surg, 13, 4, 319-326, 1986.
2. Landi A: Reconstruction of the thumb. Chapman and Hall Ltd, London, 1, 3-10, 1989.
3. Fajal G: L'Histoire du protheses et des ortheses, les grandes voies de progres. These de grade, University of Nancy, 1972.
4. Huguier PC: Considerations Anatomiques et Physiologiques pour Servir à la Chirurgie du Pouce (oeuvre posthume), Asselin, Paris, 1874.
5. Huguier PC: Remplacement du pouce par son metacarpien, par l'agrandissement du premier espace interosseux. Arch Gen Med, 1, 78 - 82, 1874.
6. Guermonprez F: Essai de cheiroplastie: tentative de restauration du pouce au moyen d'un débris de médius. Soc Chir Plast, Paris, July 28, 1886.
7. Nicoladoni C: Daumenplastik. Wien klin Wochenschr, 10, 663-665, 1897.
8. Nicoladoni C: Daumenplastik und organischer Ersatz der Fingerspitze (Anticheiroplastik und Daktyloplastik). Arch klin Chir, 61, 606-614, 1900.
9. Joyce JL: A new operation for the substitution of a thumb. Brit J Surg, 5, 499, 1918.
10. Laméris HJ: Plastiek van den duim. Ned Tijdschr Geneesk, 45, 1686, 1909.
11. Haeseker B: Dr. J.F.S. Esser and his contributions to plastic and reconstructive surgery. Thesis,

University of Rotterdam, The Netherlands, 1983.

12. Esser JFS: Operativen Ersatz der Mittelhand nebst 4 Fingern. Bruns' Beitr. Klin. Chir., 108, 244-248, 1917.

13. Esser JFS: Reconstruction of hand and 4 fingers by transplantation of middle part of foot and 4 toes. Ann Surg, 111, 655-659, 1940.

14. Krukenberg H: Über plastisches Umwertung von Amputationsstumpen. Ferdinand Enke Verlag, Stuttgart, 1917.

15. Oehlecker F: Über Zehenverpflanzung nach Nicoladoni. (Erfolgreiche Verpflanzung der großen Zehe bei Verlust der Hand samt Handwurzel.) Langenbecks Arch klin chir, 126, 436-442, 1923.

16. Monks GH: The restoration of a lower eyelid by a new method. Boston Med Surg J, 139, 385-387, 1898.

17. Esser JFS: Island Flaps. New York Med J, 106, 264-265, 1917.

18. Bunnell St: Digit transfer by neurovascular pedicle. J. Bone Joint Surg. 34A, 772-774, 1952.

19. Gosset J: La pollicisation de l'index (Technique chirurgicale). J Chir (Paris), 65, 403 - 411, 1949.

20. Littler JW: The neurovascular pedicle method of digital transposition for reconstruction of the thumb. Plast Reconstr Surg 12, 303-319, 1953.

21. Moberg E: The treatment of mutilating injuries of the upper limb. Surg Clin N Amer 44, 1107-113, 1964.

22. Halsted WS, Reichert FL, Mont-Reid: Replantations de membres entiers sans suture des vaisseaux, Lyon Chirurg, 19, 369, 1922.

23. Höpfner E: Über Gefässnaht, Gefässtransplantationen und Replantation von amputierten Extremitäten. Arch Klin Chir 70, 417, 1903.

24. Horoch C von: Die Gefässnaht, Allgemeine Wiener Medizinische Zeitung, 33, 263, 1888.

25. Murphy JB: Resection of arteries and veins injured in continuity - End-to-end suture. Exp Chir Res Med Res, 51, 73, 1897.

26. Dörfer J: Über Arteriennaht. Beitrage zur klinischen Chirurgie. 25, 781, 1899.

27. Carrel A, Guthrie CC: Anastomosis of blood vessels by the patching method and transplantation of the kidney. JAMA XLXII, 1648, 1906.

28. Carrel A, Guthrie CC: Complete amputation of the thigh, with replantation. Amer J Med Science, 131, 297, 1906.

29. Carrel A, Guthrie CC: Uniterminal and biterminal venous transplantation. Surg Gyn Obst, 2, 266, 1906.

30. Malt RA, McKhann CF: Replantation of severed arms. JAMA 189, 716, 1964.

31. Chen ZW, Chien YC, Pao YS: Salvage of the forearm following complete traumatic amputation: report of a case. Chinese Med J 82, 632, 1963.

32. Vogt B: Erfolgreiche Replantation eines vollständig amputierten Armes. Helv chir Acta 37: 230, 1970.

33. Büssner Th: Erfolgreiche Replantation eines total amputierten Armes. Spätresultat. Diss Zürich, 1978.

34. Shumacker Jr HB, Lowenberg RI: Experimental studies in vascular repair. Surg 24, 79, 1948.

35. Nylen CO: The microscope in aural surgery, its first use and later development. Acta Otolaryngologica, 116 Supp, 226, 1954.

36. Peritt RA: Recent advances in corneal surgery. In American Academy of Opthalmology and Otolaryngology, Course 280, 1950.

37. Jacobson JH, Suarez EL: Microsurgery in the anastomosis of small vessels. Surgical Forum, 11, 243, 1960.

38. Jacobson JH, Miller DB, Suarez E: Microvascular surgery: a new horizon in coronary artery

surgery. Circulation 22, 767, 1960.
39. Buncke HJ, MacLean DH: The advantage of a straight needle in microsurgery. Plast Reconstr Surg, 47, 602, 1971.
40. Acland RD: New instruments for microvascular surgery. Br J Surg, 59, 181, 1972.
41. Acland RD: Microvascular anastomosis: a device for holding stay structures and a new vascular clamp. Surg, 75, 185, 1974.
42. O'Brien C, Hayhurst JW: Metallized microsutures and a new micro needle holder. Plast Reconstr Surg, 52, 673, 1973.
43. Ikuta Y: Microvascular Surgery. Hiroshima, Lens Press, 1975.
44. Lee S: Experimental microsurgery. New York, Tokyo: Igaku-Shoin Medical Publishers Inc, 1987.
45. Buncke HJ, Schulz WP: Experimental digital amputation and reimplantation. Plast Reconstr Surg 36:1, 62, 1965.
46. Buncke HJ, Schulz WP: Total ear reimplantation in the rabbit utilizing micro-miniature vascular anastomoses. Br J Plast Surg, 19, 15, 1966.
47. Buncke HJ, Buncke CM, Schulz WP: Immediate Nicoladoni procedure in the rhesus monkey, or hallux-to-hand transplantation utilising microminiature vascular anastomoses. Br J Plast Surg, 19, 332, 1966.
48. Goldwyn RM, Lamb DL, White WL: An experimental study of large island flaps in dogs. Plast Reconstr Surg, 31, 528, 1963.
49. Krizek TJ, Tasaburo T, Desprez JD, Kiehn CL: Experimental transplantation of composite grafts by microsurgical vascular anastomoses. Plast Reconstr Surg, 36, 538, 1965.
50. Strauch B, Murray DE: Transfer of composite graft with immediate suture anastomosis of its vascular pedicle measuring less than 1 mm in external diameter using microsurgical techniques. Plast Reconstr Surg, 40, 325, 1967.
51. O'Brien C, Shanmugam M: Experimental transfer of composite free flaps with microvascular anastomoses. Australian and New Zealand J Surg, 43, 285, 1973.
52. Dong Yue Yang, Gu YD: Free second toe transplantation in reconstruction of the thumb (Report of 40 cases). Chin J Surg, 15, 13-18, 1977.
53. Antia NH, Buch VI: Transfer of an abdominal dermo-fat graft by direct anastomosis of blood vessels. Br J Plast Surg, 24, 15, 1971.
54. Cobbett JR: Free digital transfer. Report of a case of transfer of a great toe to replace an amputated thumb. J Bone Joint Surg, 51B, 677, 1969.
55. McLean BH, Buncke Jr HJ: Autotransplant of omentum to a large scalp defect with microsurgical revascularization. Plast Reconstr Surg, 49, 268, 1972.
56. Daniel RK, Taylor GI: Distant transfer of an island flap by microvascular anastomosis. A clinical technique. Plast Reconstr Surg, 52, 111, 1973.
57. O'Brien B, MacLeod AM, Miller TDH, Newing RK, Hayhurst JW, Morrison WA: Successful transfer of a large island flap from the groin to the foot by microvascular anastomoses. Plast Reconstr Surg 52, 271, 1973.
58. Ohmori K, Harii K: Free groin flaps: their vascular basis. Br J Plast Surg, 28: 238-246, 1975.
59. dos Santos LF: The scapular flap: A new microsurgical free flap. Bol Chir Plast 70, 133, 1980.
60. Barwick WJ, Goodkind DJ, Serafin D: The free scapular flap. Plast Reconstr Surg, 69, 779, 1982.
61. Urbaniak JR, Koman LA, Goldner RD: The vascularized cutaneous scapular flap. Plast Reconstr Surg, 69, 772, 1982.
62. Massif TM, Vidal L, Bover JL, Bander J: The parascapular flap: a new cutaneous microsurgical free flap. Plast Reconstr Surg, 69, 591, 1982.
63. Fissette J, Lahaye T, Colot G: The use of the free parascapular flap in midpalmar soft tissue defect.

Ann Plast Surg, 10, 235, 1983.

64. Yang G, Chen B, Gao Y: Forearm free skin flap transplantation. Natl Med J China, 61, 139, 1978.

65. Muhlbauer W, Herndl E, Stock W: The forearm flap. Plast Reconstr Surg, 70, 336, 1982.

66. Soutar DS, Scheker LR, Tanner NSB: The radial forearm flap: a versatile method for intra-oral reconstruction. Br J Plast Surg, 36, 1, 1983.

67. Hovius SER, Sluimers JE, Adrichem LNA van, Vaandrager JM, Wijthoff SJM, Meulen JC van der: The radial forearm flap. Neth J Surg, 40-3, 69, 1988.

68. Song Ruyao, Song Yeguang, Yu Yuseng, Song Yeliang: The upper arm flap. Clin Plast Surg, 9, 27, 1982.

69. Katsaros J, Schusterman M, Beppu M, Bannis JC, Acland RD: The lateral upper arm flap: anatomy and clinical applications. Ann Plast Surg, 6, 489, 1984.

70. Daniel RK, Terzis J, Midgley R: Restoration of sensation of an anesthetic hand by a free neurovascular flap from the foot. Plast Reconstr Surg, 57, 275, 1976.

71. Gilbert A: Composite tissue transfers from the foot: anatomic basis and surgical technique. In: Daniller A, Strauch B (eds) Symposium on Microsurgery, 15, 25, 1976.

72. McGraw JB, Furlow LT: The dorsalis pedis arterialized flap. A clinical study. Plast Reconstr Surg, 55, 177, 1975.

73. Robinson DW: Microsurgical transfer of the dorsalis pedis neurovascular island flap. Br J Plast Surg, 29, 209, 1976.

74. Maxwell PG: Iginio Tansini and the origin of the latissimus dorsi musculocutaneous flap. Plast Reconstr Surg, 65, 686, 1980.

75. Olivari N: The latissimus flap. Br J Plast Surg, 29, 126, 1976.

76. Bostwick J, Nahai F, Wallace JG, Vasconez LO: Sixty latissimus dorsi flaps. Plast Reconstr Surg, 63, 31, 1979.

77. Harii K, Ohmori K, Torii S: Free gracilis muscle transplantation with microneurovascular anastomoses for the treatment of facial paralysis: A preliminary report. Plast Reconstr Surg, 57, 133, 1976.

78. Manktelow RT, Zuker RM, McKee NH: Functioning free muscle transplantation. J Hand Surg, 9A, 32, 1984.

79. Pennington D, Lai M, Pelly A: The rectus abdominus myocutaneous free flap. Br J Plast Surg, 33, 277, 1980.

80. Cunningham B, Christiansen M, Dressel T, Shons A: The rectus abdominus muscle: a useful free flap donor site. Presented at the American Society of Plastic and Reconstructive Surgeons Annual Meeting, Honolulu, October 1982.

81. Taylor GI, Corlett R, Boyd JB: The extended deep inferior epigastric flap: a clinical technique. Plast Reconstr Surg, 72, 751, 1983.

82. Soutar DS, McGregor IA: The radial forearm flap in intraoral reconstruction: the experience of 60 consecutive cases. Plast Reconstr Surg, 78, 1, 1986.

83. Biemer E, Stock W: Total thumb reconstruction: a one-stage reconstruction using an osteo-cutaneous forearm flap. Br J Plast Surg, 36, 52, 1983.

84. Swartz WM, Banis JC, Newton ED, Ramasastry SS, Jones NF, Acland R: The osteocutaneous scapular flap for mandibular and maxillary reconstruction. Plast Reconstr Surg, 77, 4, 530, 1986.

85. Teot L, Bosse JP, Moufarrege R, Papillon J, Beauregard G.: The scapular crest pedicled bone graft. Int J Microsurg, 3, 257, 1981.

86. Taylor GI, Watson N: One-stage repair of compound leg defects with free, revascularized flaps of groin skin and iliac bone. Plast Reconstr Surg, 61, 494, 1978.

87. Taylor GI: Reconstruction of the mandible with free composite iliac bone grafts. Ann Plast Surg, 9,

361, 1982.
88. Taylor GI, Miller GDH, Ham FJ: The free vascularized bone graft: A clinical extension of microvascular techniques. Plast Reconstr Surg, 55, 533, 1975.
89. Gilbert A: Vascularized transfer of the fibula shaft. Int. J. Microsurg, 1, 100, 1979.
90. Buncke HJ, McLean DH, George PT: Thumb replacement: great toe transplantation by microvascular anastomoses. Br J Plast Surg, 26, 194, 1973.
91. May JW, Daniel RK: Great toe to hand free tissue transfer. Clin Orthop, 133, 140, 1978.
92. O'Brien B, MacLeod AM, Sykes PJ: Hallux-to-hand transfer. Hand, 7, 2, 1975.
93. Chang Ti-Shang, Wang Wei, Wu Jiu-Bao: Free transfer of the second toe combined with dorsalis pedis flap using microvascular technique for reconstruction of the thumb and other fingers. Ann Ac Med Singapore, 8, 404, 1981.
94. Leung PC: Thumb reconstruction using second-toe transfer. Hand Clin, 1, 2, 285, 1985.
95. O'Brien B, Brennen M, MacLeod AM: Simultaneous double toe transfer for severely disabled hands. Hand, 10, 232, 1978.
96. Tsai TM: Second and third toe transplantation to a transmetacarpal amputated hand. Ann Ac Singapore 8, 413, 1979.
97. Tsai TM, Jupiter JB, Wolf T, Atasoy E: Reconstruction of severe transmetacarpal mutilating hand injury by combined second and third toe transfer. J Hand Surg, 6, 319, 1981.
98. Morrison WA, O'Brien B, MacLeod AM: Thumb reconstruction with a free neurovascular wrap-around flap from the big toe. J Hand Surg, 5, 575, 1980.
99. O'Brien B: Microvascular free small joint transfer. Microvascular Reconstructive surgery. Churchill Livingstone, Edinburgh, 284, 1977.
100. Tsai TM, Jupiter JB, Kutz JE, Kleinert HE: Vascularized autogenous whole joint transfer in the hand - a clinical study. J Hand Surg, 7, 335, 1982.
101. Reid CD, Moss ALH: One-stage flap repair with vascularised tendon grafts in a dorsal hand injury using the "Chinese" forearm flap. Br J Plast Surg, 36, 473, 1983.
102. Kleinert HE, Kasdan ML: Salvage of devascularized upper extremities including studies on small vessel anastomosis. Clin Orthop, 29, 29, 1963.
103. Komatsu S, Tamai S: Successful replantation of a completely cut-off thumb. Plast Reconstr Surg, 42, 374, 1968.
104. Sixth People's Hospital, Shanghai. Reattachment of traumatic amputations. A summing up of experience. Chin Med J, I, 392, 1967.
105. O'Brien B, MacLeod AM, Miller TDH, Newing RK, Hayhurst JW, Morrison WA: Clinical implantation of digits. Plast Reconstr Surg, 53, 490, 1973.
106. Ikuta Y et al: The reattachment of severed fingers. Hiroshima J Med Sciences, 22, 131, 1973.
107. Kleinert HE: Replantation of non-viable digits - ten year experience. J Bone Joint Surg, 56A, 1092, 1975.
108. Biemer E, Duspiva W, Herndl E, Stock W, Ramatschi P: Early experiences in organising and running a replantation service. Br J Plast Surg, 31, 9, 1977.
109. Biemer E, Duspiva W, Stock W: Report of a Replantation service. Experiences and results of 407 replantations. In: transactions of the 7th International Congress of International Society for Plastic and Reconstructive Surgery. Rio de Janeiro, Cartgraf, Sao Paulo, Brazil, 75, 1979.
110. Tamai S: Digital replantation. Clin Plast Surg, 5, 195, 1978.
111. Smith JW: Microsurgery of peripheral nerves. Plast Reconstr Surg, 33, 317, 1964.
112. Michon J, Masse P: Le moment optimum de la suture nerveuse dans les plaies on membre supérieur. Rev Chir. Orthop. et Reparatrice de l'Appareil Moteur, 50, 205, 1964.
113. Bora FW, Pleasure DE, Didizion NA: A study of nerve regeneration and neuroma formation after

nerve suture by various techniques. J Hand Surg, 1, 138, 1976.

114. Cabaud HE, Rodkey WG, McCarroll HR, Mutz S, Neibauer JE: Epineurial and perineurial fascicular nerve repairs: a critical comparison. J Hand Surg, 1, 131, 1976.

115. Cabaud HE, Rodkey WG, McCarroll HR: Peripheral nerve injuries: studies in higher non-human primates. J Hand Surg, 5, 201, 1980.

117. Millesi H, Meissl G, Berger A: Further experience with interfascicular grafting of the median, ulnar and radial nerves. J Bone Joint Surg, 58a, 2209, 1976.

118. Lundborg G: Nerve injury and repair. Churchill Livingstone, Edinburgh, London, Melbourne, New York, 1988.

119. MacKinnon SE, Dellon AL: Surgery of the peripheral nerve. Thieme Medical Publishers Inc, New York, 1988.

120. Terzis JK (ed): Microreconstruction of nerve injuries. Philadelphia U.S.A.: WB Saunders Co, 1987.

121. Kleinman WB, Putman MD: Microvascular free-tissue transfers to the hand and upper extremity. Hand Clin, 5, 3, 423, 1989.

122. Ikuta Y: Vascularized free flap transfer in the upper limb. Hand Clin, 1, 2, 297, 1985.

123. O'Brien B, Morrison WA: Reconstructive Microsurgery. Churchill Livingstone, Edinburgh, London, Melbourne, New York, 1987.

124. Buncke HJ: Microsurgery: transplantation - replantation. Lea & Febiger, Philadelphia, London, 1991.

125. Urbaniak JR: Wrap-around procedure for thumb reconstruction. Hand Clin, 1, 2, 259, 1985.

126. May JW, Bartlett SP: Great toe-to-hand free tissue transfer for thumb reconstruction. Hand Clin, 1, 2, 271, 1985.

127. Leung PC: Thumb reconstruction using second-toe transfer. Hand Clin, 1, 2, 285, 1985.

128. Gilbert A: Reconstruction of congenital hand defects with microvascular toe transfers. Hand Clin, 1, 2, 351, 1985.

129. Wei FC, Colony LH: Microsurgical reconstruction of opposable digits in mutilating hand injuries. Clin Plast Surg, 16, 3, 491, 1989.

130. Dijkstra R, Bos KE: Functional results of thumb reconstruction. The Hand, 14, 2, 120, 1982.

131. Harashina T: Analysis of 200 free flaps. Br J Plast Surg, 41, 33, 1988.

132. O'Brien B: The role of microsurgery in modern surgery. Ann Plast Surg, 24, 258, 1990.

133. Khouri RK, Shaw WW: Reconstruction of the lower extremity with microvascular free flaps: a 10-year experience with 304 consecutive cases. J Trauma, 29, 1086, 1989.

134. Jones B, Smith P, Harrison D: Replantation. Br Med J, 287, 6384, 1, 1983.

135. Weiland AJ, Villarreal-Rios A, Kleinert HE, Kutz J, Atasoy E, Lister G: Replantation of digits and hands: analysis of surgical techniques and functional results in 71 patients with 86 replantations. J Hand Surg, 2, 1, 1, 1977.

136. Tamai S: Twenty years experience of limb replantation - review of 293 upper extremity replants. J Hand Surg, 7, 6, 549, 1982.

137. Buck-Gramcko D: Indikation und Technik der Replantation. Langenbecks Arch Chir, 347, 97, 1978.

138. Chen Chung Wei, Yun-Qing Q, Zhong-Jia Y: Extremity replantation. World J Surg, 2, 513, 1978.

139. Jones MB: Monitors for the cutaneous microcirculation. Plast Reconstr Surg 73(5); 843-850, 1984

140. Gerritsen J: De revalidatie van patiënten na replantatie, revascularisatie of uitgebreide reconstructie van delen van de arm. Ned Tijdschr Geneesk, 129, 31, 1469, 1985.

141. Kader PB: Therapists management of the replanted hand. In: Hunter, Schneider, Mackin, Callahan (eds): Rehabilitation of the hand - surgery and therapy part VIII, Replantation, 63, 821, 1990.

142. Petrilli J, Milne EJ, Nugent K, Kaplan R, Lineaweaver WC: Hand Therapy. In: Buncke HJ (Ed): Microsurgery: transplantation - replantation, Lea & Febiger, Philiadelphia, London, 42, 748, 1991.

143. Meyer VE: Hand amputations proximal but close to the wrist joint: prime candidates for reattachment (long-term functional results). J Hand Surg, 10A, 6, 989, 1985.

144. No. VI People Hospital, Shanghai: Replantation of severed limbs retrospect and prospect. Chin Med J, 1, 3, 1973.

145. Meyer VE: Beitrag zur Indikation und Technik der mikrochirurgischen Revaskularisation im Vorderarm Handbereich. Z Unfallmed Berufskr 75, Heft 1, 1982.

146. Jones JM, Schenck RR, Chesney RB: Digital replantation and amputation - comparison of function. J Hand Surg, 7, 2, 183, 1982.

147. May JW, Toth BA, Gardner M: Digital replantation distal to the proximal interphalangeal joint. J Hand Surg, 7, 161, 1982.

148. Scott FA, Howar JW, Boswick JA: Recovery of function following replantation and revascularization of amputated hand parts. J Trauma, 21, 3, 204, 1981.

149. Chen ZW, Meyer VE, Kleinert HE, Beasley RW: Present indications and contraindications for replantation as reflected by long-term functional results. Orthop Clin North Am, 12, 849, 1981.

150 Dellon AL: Sensory recovery in replanted digits and transplanted toes: a review. J Reconstr Microsurg, 2, 2, 123, 1986.

151. Yamauchi S, Nomura S, Yoshimura M, Ueno T, Iwai Y, Shimamura K: A clinical study of the order and speed of sensory recovery after digital replantation. J Hand Surg, 8, 5, 545, 1983.

152. Kort WJ, Wolff ED, Eastham WN: Heterotopic auxiliary liver transplantation in rats. Transpl, 12, 6, 415, 1971.

153. Tolhurst DE, Bos KE: Free revascularized muscle grafts in facial palsy. Plast Reconstr Surg, 5, 760, 1982.

154. Smith AR, Urk H van, Kort W, Wijthoff SJM, Sonneveld GJ, Bos KE, Meulen JC van der: Replantaties en revascularisaties van meer dan 100 vingers, handen en benen in het Academisch Ziekenhuis Rotterdam - Dijkzigt. Ned Tijdschr Geneesk 126, 35, 1574, 1982.

155. Smith AR, van Alphen WA, Faithfull NS, Fennema M: Limb preservation in replantation surgery. Plast Reconstr Surg, Feb, 227, 1985.

156. Smith AR, Sonneveld GJ, Kort WJ, van der Meulen JC: Clinical application of transcutaneous oxygen measurements in replantation surgery and free tissue transfer. J Hand Surg 8, 2, 139, 1983.

157. Clinton MS, Sepka RS, Bristol D, Pederson WC, Barwick WJ, Serafin D, Klitzman B: Establishment of Normal Ranges of Laser Doppler Blood Flow in Autologous Tissue Transplants. Plast Reconstr Surg, Feb, 299, 1991.

158. Hovius SER, Adrichem LNA van, Mulder HD, Strik R van, Meulen JC van der: The predictive value of the laser doppler flowmeter for postoperative microvascular monitoring. Submitted to the Ann Plast Surg.

159. Smith AR, van de Werken L, Wijers O, van Alphen WA, van der Meulen JC: Kosten-baten analyse in de replantatiechirurgie. Ned Tijdschr Geneesk 129, 31, 1466, 1985.

160. JE Sluimers (plastic surgeon in The Hague, previously working in Rotterdam): personal communication

161. Letsel van de bovenste extremiteit. Privé Ongevallen Registratie Systeem PORS, Stichting Consument en Veiligheid, Hobbemastraat 22, Amsterdam.

162. Accident facts. National Safety Council, Chicago, 1987.

163. Occupational finger injuries - United States 1982. Morbid Mortal Week Rep. 32(45), 589, 1983.

164. Kelsey JL, Pastides H, Kreiger N, Harris C, Chernow RA: Upper extremity disorders; a survey of their frequency and cost in the United States. The CV Mosby Company, St. Louis / Toronto / London, 1980.

165. Burke FD, Dias JJ, Lunn PG, Bradley M: Providing care for hand disorders: trauma and elective. Br

J Hand Surg, 16B, 1, 13, 1991.
166. Blair SJ, Bear-Lehman J, McCormick E: Industrial hand injuries: prevention and rehabilitation. In: JM Hunter (Ed) - Rehabilitation of the hand, surgery and therapy. The C.V. Mosby Company, St. Louis, Baltimore, Philadelphia, Toronto, 1990.
167. Nylander G, Vilkki S, Östrup L: The need for replantation surgery after traumatic amputations of the upper extremity - an estimate based upon the epidemiology of Sweden. J Hand Surg, 9B, 3, 257, 1984.
168. Stichting Informatie Gezondheidszorg, Maliebaan 50, 3581 CS Utrecht.
169. Yu ZJ, Wang Y: Reconstruction of the "Hand" - a case report. Nat Med J China, 59, 593, 1979.
170. Yu ZJ, Ho HG: Bilateral hand reconstruction: report of three cases. J Reconstr Microsurg, 1, 253, 1985.
171. Vilkki SK: Freie Zehenubertragung auf den Unterarmstumpf nach Handgelenkamputation - eine moderne Alternative zur Krukenberg-Operation. Handchir Mikrochir Plast Chir, 17, 92, 1985.
172. Swanson AB, Swanson GC: The Krukenberg procedure in the juvenile amputee. Clin Orthop, 148, 55, 1980.
173. Murphy EF: Commentary. In Selected articles from Artif. Limbs, Huntington, NY, RE Krieger Publishing Co Inc, 1970.
174. Dembo T, Leviton GL, Wright BA: Adjustment to misfortune: a problem of social-psychological rehabilitation. In Selected articles from Artif. Limbs, Huntington, NY, RE Krieger Publishing Co Inc, 1970.
175. Billock JN: Prosthetic management of complete hand and arm deficiencies. In: Rehabilitation of the hand. 85, 1072. Ed. JM Hunter. The CV Mosby Co, St. Louis/Baltimore/Philadelphia/Toronto, 1990.
176. Childress DS, Holmes DW, Billock JN: Ideas on myoelectric prosthetic systems for upper-extremity amputees. In: Herberts P and others eds.: The control of upper-extremity prosthesis and orthoses. Springfield, Ill, Charles C Thomas, Publ, 1974.
177. Malone JM et al: Immediate early and late post-surgical management of upper-limb amputations. J Rehabil R and D, 21(1), 33, 1984.
178. Erhardt RP: Sequential levels on development of prehension. Am J Occup Ther, 28, 592, 1975.
179. Reiter R: Feine Neue Electrokunsthand. Grenz Med, 4, p 133, 1948.
180. Le Blanc MA: Patient population and other estimates of prosthetics and orthotics in the USA. Ortho Prosthet, 27(3), 38, 1973.
181. Billock JN: Upper limb prosthetic terminal devices: hands versus hooks. Clin Orthot Prosthet, 10(2), 57, 1986.
182. Taylor CL, Schwarz RJ: The anatomy and mechanics of the human hand. In Selected Articles from Artif. Limbs, Huntington, NY, RE Krieger Publ Co Inc, 1970.
183. Taylor CL.: Biomechanics of control. In Selected Articles from Artif. Limbs., Huntington, NY, RE Krieger Publ Co Inc, 1970.
184. Billock JN: Upper limb prosthetic management - hybrid design approaches. Clin Orthot Prosthet, 9(1), 23, 1985.
185. Sauter WF: Myoelectric and switch controlled upper extremities prostheses. Amputation surgery and rehabilitation. The Toronto experience, New York, Churchill Livingstone, 1981.
186. Rose EH: The high-tech hand surgery interface. Clin Plast Surg, 13, 2, 333, 1986.
187. Pillet J, Mackin EJ: Aesthetic hand prosthesis: its psychologic and functional potential. In: Rehabilitation of the hand. 82, 1039. Ed. JM Hunter. The CV Mosby Co, St. Louis/Baltimore/Philadelphia/Toronto, 1990.
188. Murray JE: Current evaluation of human kidney transplantation. Editorial, Plast Reconstr Surg, 34,

1, 93, 1964.

189. Hall RH: Whole upper extremity transplant for human beings: general plans of procedure and operative technic. Ann Surg, 120, 1, 12, 1944.

190. Cited in: Goldwyn RM, Beach PM, Feldman D, Wilson RE: Canine Limb Homotransplantation. Plast Reconstr Surg, 37, 3, 184, 1966.

191. Usui M, Ishii S, Muramatsu I, Takaihata N: An experimental study on Replantation toxaemia and the effect of hypothermia on an amputated limb. J Hand Surg, 3, 589, 1978.

192. Eiken O, Mayer RF, Nabseth DC, Deterling RA: Limb replantation. II: The pathophysiological effects. Arch Surg, 88, 54, 1964.

193. Lundborg G: Ischemic nerve injury. Experimental studies on intraneural microvascular pathophysiology and nerve function in a limb, subjected to temporary circulatory arrest. Scand J Plast Reconstr Surg, Suppl 1-113, 1970.

194. Chiu HY, Chen AT: Revascularizations of digits after 33 hours of warm ischemia time: a case report. J Hand Surg, 9A, 63, 1984.

195. May JW, Hergrueter CA, Hansen RH: Seven digit replantation: digit survival after 39 hours of cold ischemia. Plast Reconstr Surg, 78, 522, 1986.

196. Alphen WA van, Smith AR, Kate FJW ten: Maximum hypothermic ischemia in replants containing muscular tissue. J Hand Surg, 13a, 427, 1988.

197. Collins GH, Bravo-Shugarman MB, Teraski PI: Kidney preservation for transportation. Lancet 2, 1219, 1969.

198. Belzer FO, Ashby BS, Dumphy JE: 24-hour and 72-hour preservation of canine kidneys. Lancet, 2, 536, 1967.

199. Belzer FO, Southard JH: Principles of solid-organ preservation by cold storage. Transpl 45, 4, 673, 1988.

200. Southard JH, Marsh DC, McAnulty JF, Belzer FO. Oxygen-derived free radical damage in organ preservation: activity of superoxide dismutase and xanthine oxidase. Surg, 101, 566, 1987.

201. Todo S, Nery J, Yanaga K, Podesta L, Gordon RD, Starzl TE: Extended preservation of human liver grafts with UW solution. JAMA, 261 (feb 3), 711, 1989.

202. Southard JS: Advances in organ preservation. Transpl Proc, 21, 1 (february), 1195, 1989.

203. Ploeg RJ, Goossens D, Mc Anulty JF, Southard JH, Belzer FO: Efficacy of cytoprotective agents on the function of the hypothermically preserved dog kidney during normothermic reperfusion. Transpl Proc 21(1), 1247-49, 1989.

204. Faithfull NS, Fennema M, Erdmann W, et al; Tissue oxygenation by fluorocarbons. In: Proceedings of the International Society for Oxygen Transport to Tissue. Rushton, Los Angeles 16-19 August 1983.McAnulty JF, Ploeg RJ, Vreugdenhil P et al: Transpl. Proc. 1988.

205. Smith AR, Alphen WA van, Faithfull S, Fennema M: Limb preservation in replantation surgery. Plast Reconstr Surg, 75, 2, 227, 1985.

206. Adams DS, Ponsford S, Gunson B, Boon A, Honigsberger L et al: Neurological complications following liver transplantation. Lancet, no 1., p 949, 1987.

207. Rubin AM, Kang H: Cerebral blindness and encephalopathy with cyclosporin A toxicity. Neurology, 37, 1072, 1987.

208. Atkinson K, Biggs J, Darveniza P, Boland J, Concannon A et al: Cyclosporine-associated central nervous system toxicity after allogeneic bone marrow transplantation. Transpl, 38, 34, 1984.

209. Scott JP, Higenbottam TW: Adverse Reactions and Interactions of Cyclosporin. Med Tox, 3, 107, 1988.

210. Bain JR, Mackinnon SE, Hudson AR, Falk RE, Falk JA, Hunter DA: The peripheral nerve allograft:

a dose-response curve in the rat immunosuppressed with cyclosporin A. Plast Reconstr Surg 82/3, 447, 1988.

211. Bain JR, Mackinnon SE, Hudson AR, Falk RE, Falk JA, Hunter DA: The peripheral nerve allograft: an assessment of regeneration across nerve allografts in rats immunosuppressed with CyA. Plast Reconstr Surg 82/6, 1052, 1988.

212. Mackinnon SE, Hudson AR, Gentili F, Kline DG, Hunter D: Peripheral nerve injection injury with steroid agents. Plast Reconstr Surg 69: 482, 1982.

213. Samulack DD, Dykes RW, Munger BL: Neurophysiologic aspects of allogeneic skin and upper extremity composite tissue transplantation in primates. Transpl Proc, XX, 2, suppl 2, 279, 1988.

214. Akveld JEM: Orgaantransplantatie en het toestemmingssysteem. Med Contact, 29, 875, 1985.

215. Boer EJ: Het tekort aan donororganen: haken en ogen aan wetgeving en praktijk rond het afstaan van organen voor transplantatie. Ned Tijdschr Geneesk 133, 2571, 1989.

216. Leeuwen E van: Marktonderzoek orgaandonatie. Succesfactoren en knelpunten van de orgaandonatieprocedure. Leiden: Stichting Eurotransplant, 1989.

217. Bunnell S: Surgery of the Hand. JB Lippincott company, Philadelphia, 1, 1944.

218. Ashley-Montagu FM: On the primate hand. Amer Jour Phys Anthropol, 15, 291-314, jan-mar, 1931.

219. Napier JR: Prehensility and opposability in the hands of primates. Symp Zool Soc Lond, 5, 115-132, 1961.

220. Ducker TB: Pathophysiology of peripheral nerve trauma. In: Omer GE, Spinner M (eds): Management of peripheral nerve problems. WB Saunders, Philadelphia, 476, 1980.

221. Horch K: Central responses of cutaneous sensory neurons to peripheral nerve crush in the cat. Brain Res, 151, 581, 1978.

222. Kristensson K: Retrograde signalling of nerve cell body response to trauma. In Gorio A, Millesi G, Mingrino S (eds.): Posttraumatic peripheral nerve regeneration. Raven Press, New York, 27, 1981.

223. Yamada KM, Spooner BS, Wessells NK: Ultrastructure and function of the growth cones and axons of cultured nerve cells. J Cell Biol 49, 614, 1971.

224. Ivins JK, Pittman RN: Growth cone-growth cone interactions in cultures of rat sympathetic neurons. Dev Biol, 135, 147, 1989.

225. Waller AV: Experiments on the glossopharyngeal and hypoglossal nerves of the frog and observations produced thereby in the structure of their primative fibres. Phil Trans R Soc Lond, 140, 423, 1850.

226. Gorio A, Carmignoto G: Reformation, maturation and stabilization of neuromuscular junctions in peripheral nerve regeneration. In Gorio A, Millesi H, Mingrino S (eds.): Posttraumatic peripheral nerve regeneration. Raven Press, New York, 481, 1981.

227. Karpati G, Carpenter S, Sharoon L: Experimental reinnervation attempts of skeletal muscle cells by nonmotor nerves. In Gorio A, Millesi H, Mingrino S (eds.): Posttraumatic peripheral nerve regeneration. Raven Press, New York, 495, 1981.

228. Mackinnon SE, Dellon AL, Hudson AR, Hunter DA: Alteration of neuroma formation by manipulation of neural environment in primates. Plast Reconstr Surg, 76, 345, 1985.

229. Kirklin JW, Murphy F, Berkson J: Suture of peripheral nerves: factors affecting prognosis. Surg Gynecol Obstet, 88, 719, 1949.

230. Oester VT, Davis L: Recovery of sensory functions. In Woodhall B, Beebee GW (eds): Peripheral nerve regeneration, U.S. Government Printing Office, Washington DC, 241, 1956.

231. Davis L: The return of sensation to transplanted skin. Surg Gynecol Obstet, 59, 533, 1934.

232. Kredel FE, Evans JP: Recovery of sensation in denervated pedicles and free skin grafts. J Neurol Neurosurg Psych 19, 1203, 1933.

233. Adeymo O, Wyburn GN: Innervation of skin grafts. Trans Bull, 4, 152, 1957.

234. Mannerfelt L: Evaluation of functional sensation of skin grafts in the hand area. Br J Plast Surg, 15, 136, 1962.
235. Olsson Y, Kristensson K, Klatzo J: Permeability of vasa nervorum and perineurium in mouse sciatic nerve studies by fluorescence and electronmicroscopy. J Neuropathol Exp Neurol, 30, 105, 1971.
236. Smith BH, Kornblith PL: Axoplasmic transport in neurological surgery. Neurosurg, 10, 268, 1982.
237. Droz B, Rambourg A, Koenig HL: The smooth endoplasmic reticulum. Structure and role in the renewal of axonal membrane and synaptic vesicles by fast axonal transport. Brain Res, 93, 1, 1975.
238. Ochs S: Basic properties of axonplasmic transport. In Dyck PJ, Thomas PK, Lambert EH, Bunge R (eds.): Peripheral Neuropathy, 2nd ed., WB Saunders, Philadelphia, 1984.
239. Lasek RJ, McQuarrie IG, Wujek JR: The central nervous system regeneration problem. Neuron and environment. In Gorio A, Millesi H, Mingrino S (eds.): Posttraumatic peripheral nerve regeneration. Raven Press, New York, 59, 1981.
240. Forssman J: Über de Ursachen welche die Wachstufstrichtung der peripheren Nervenfasern die der Regeneration bestimmen. Beitr Pathol Anat, 24, 55, 1898.
241. Mackinnon SE, Dellon AL, Lundborg G, Hudson AR: A study of neurotropism in the primate model. J Hand Surg [Am], 11, 888, 1986.
242. Brushart TM: Selective reinnervation of distal motor stumps by peripheral motor axons. Exp Neurol, 97, 289, 1987.
243. Seckel BR, Ryan SE, Gagne JR, Tin Ho Chiu CST, Watkins E: Target specific nerve regeneration through a nerve guide in the rat. Plast Reconstr Surg, 78, 793, 1986.
244. Mackinnon SE, Dellon AL: Reply to T. Brushart's letter on The study of neurotrophism in a primate model. J Hand Surg, 12A, 809, 1987.
245. Badalamente MA, Hurst LC, Paul SB, Stracher A: Enhancement of neuromuscular recovery after nerve repair in primates. J Hand Surg, 12B, 2, 211, 1987.
246. Medinaceli L de, Wyatt RJ, Freed WJ: Peripheral nerve reconnection: mechanical, thermal and ionic conditions that promote the return of function. Exp Neurol, 81, 469, 1983.
247. Dekker AJAM: Enhancement of peripheral nerve regeneration in the rat by a previous nerve injury and by treatment with neuropeptides. Thesis. Univ of Utrecht, The Netherlands, 1987.
248. Verhaagen J: Morphological and biochemical aspects of the neurotrophic action of melanocortins. Thesis. Univ of Utrecht, The Netherlands, 1987.
249. Koning P de: Functional and electrophysiological evaluation of damaged peripheral nerve: neurotrophic actions of ORG 2766. Thesis. Univ of Utrecht, The Netherlands, 1987.
250. LeBeau JM: Schwann cell-axon interactions during rat peripheral nerve regeneration and remyelination. Thesis. San Diego: Univ of California, USA, 1986.
251. Medinaceli L de: Functional consequences of experimental nerve lesions: effects of time, location and extent of damage. Exp Neurol, 100, 154, 1988.
252. Millesi H: The nerve gap. Hand Clin, 2, 651, 1986.
253. Scaravilli F: The influence of distal environment on peripheral nerve regeneration across a gap. J Neurocyt, 13, 1027, 1984.
254. Millesi H: Bedeutung der Nerventransplantation in der Chirurgie der peripheren Nerven. Zentralblatt für Chirurgie, 100, 1537, 1975.
255. Gilbert A, Merle M: Evaluation expérimentale du collage des nerfs périphériques. Communication Congrès du GAM, Lyon, Juin 1989.
256. Palazzi S: The use of fibrin in nerve adhesions. In: Schlag S, Redl H (eds): Fibrin sealant in Operative Medicine. Ophtalmology-Neurosurgery, 2, Springer-Verlag, Berlin-Heidelberg, 186, 1986.
257. Jabaley ME: Technical aspects of peripheral nerve repair. J Hand Surg, 9B, 1, 14, 1984.

258. Mackinnon SE, Dellon AL, Hudson AR, Hunter DA: Nerve regeneration through a pseudosynovial sheath in a primate model. Plast Reconstr Surg, 75, 6, 833, 1985.
259. Mackinnon SE, Dellon AL: A comparison of nerve regeneration across a sural nerve graft and a vascularized pseudosheath. J Hand Surg, 13A, 6, 935, 1988.
260. Marsh D: The validation of measures of outcome following suture of divided peripheral nerves supplying the hand. J Hand Surg, 15B, 1, 25, 1990.
261. Robinson PH: Artificial conduits in reconstructive microsurgery. Thesis, Univ Groningen, The Netherlands, 1989.
262. Merzenich MM, Kaas JH, Wall J et al: Progression of change following median nerve section in the cortical representation of the hand in areas 3b and 1 in adult owl and squirrel monkeys. Neuroscience, 10, 639, 1983.
263. Dykes RW: Central consequences of peripheral nerve injuries. Ann Plast Surg, 13, 5, 413, 1984.
264. Iggo A: Cutaneous Receptors and their sensory functions. J Hand Surg, 9B, 1, 7, 1984.
265. Dellon AL: Evaluation of sensibility and reeducation of sensation in the hand. John D Lucas Printing Co., Baltimore, 1988.
266. Samulack DD: Reinnervation of allografted primate upper-extremity tissues in the presence of cyclosporine immunosuppression. Thesis, McGill University Montreal, 1990.
267. Sethi RK, Thompson LL: The electromyographer's handbook. Second Ed., Little, Brown and Company, Boston/Toronto, 1989.

CHAPTER I: INTRODUCTION
SECTION B: Immunological aspects

H.P.J.D. Stevens

B.1 Historical notes on the role of plastic surgeons in the field of transplantation immunology

In their search for ways to reconstruct tissue defects in patients, plastic surgeons helped to launch the field of transplantation surgery. The first description of a skin transplantation, performed to repair a nose, is found in the Indian medical handbook *'Sushrutta Samheta'* dated A.D. 600[1]. In the 16th century *Gaspare Tagliacozzi*, the "Father of Plastic Surgery", was one of the first Europeans to thoroughly describe his efforts with regard to nasal reconstruction[2]. His work also generated some fanciful reports of allografting by innovative writers of his day. In exchange for his freedom, a 'sympathetic' slave was reported to have donated a parabiotic flap from his arm to a gentleman who had lost his nose in a sword-play. The reconstructed nose was a success for three years, but then the allogeneic flap was suddenly rejected, leaving the patient with his original defect. Strikingly, the slave had died of a common disease at exactly the same time. According to Tommaso Campanella, a philosopher, the slave's soul had remained as a whole, hosted in the slave's body as well as in his donated part; At death, the soul left both residences, resulting in rejection of the transplant[3]. Thereafter, successful allografts were reported from time to time through history, but none were substantiated[4]. In 1804, *Baronio* reported successful auto- and allotransplantation of skin of a sheep (fig. 1)[5]. He described the reconstruction of a nose with a skin graft taken from the thigh of a patient. However, transplantation of organs other than skin was not possible before it was known how to make anastomoses of vessels.

Genuine surgical technique in macrovascular surgery was only developed during the beginning of this century, mainly by *Carrel* and *Guthrie*. Like *Hopfner*[6] they were able to perform an autoreplantation of a dog leg[7,8]. They also transplanted the kidney unilaterally and bilaterally and, uniquely, the entire side of a dog's head including the scalp and the ear on the external carotid system. In 1922, *Guthrie* even transplanted an entire dog's head into the neck of another dog at the University of Pittsburgh and was able to demonstrate eye lid function and salivation in the transplanted head. *Padgett* and *Brown* both established that skin grafts exchanged between identical twins would survive indefinitely[9,10]. At that time allogeneic skin grafts were only found to be effective to close wounds temporarily, for example for patients with life-threatening loss of skin due to massive burns[10]. Research into the immunological nature of the 'take' or 'rejection' of skin grafts became more substantial with the work of *Medawar*, who in initial experiments collaborated with plastic surgeon *Gibson* at the Glasgow Royal Infirmary[11] during World War II. It was demonstrated that rejection characteristics such as latency, memory (second-set phenomenon), and specificity of graft rejection proved the existence of an active immune response.
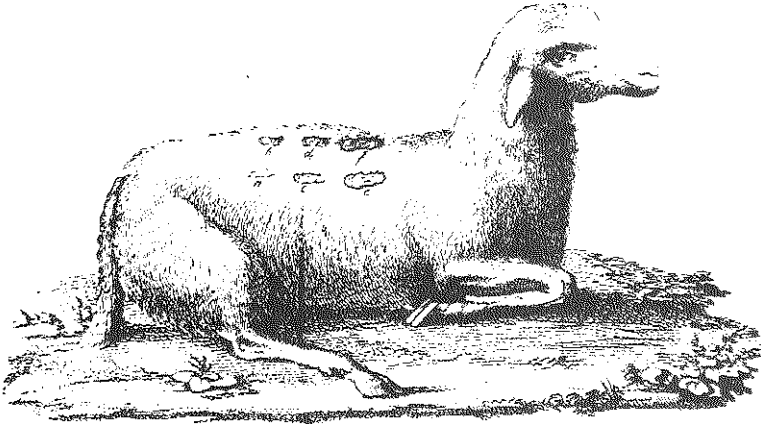
Fig. 1. The first transplantation of allogeneic skin in history, performed in a sheep (*Baronio*, 1804).

In the following decade, *Converse*, who also worked in the field of skin grafting[12,13], started the biannual International Conferences on Transplantation in New York which developed in to the International Transplantation Society[14]. Interestingly, the organizational efforts of another plastic surgeon named *Conway* who investigated skin allografts in rats with an in vivo transparent chamber[15], led to the onset of the *Transplantation Bulletin* as part of the journal, *Plastic and Reconstructive Surgery*. It was this *Transplantation Bulletin* that finally evolved into the journal *Transplantation*.

In the mid 1950's, *Murray* a plastic surgeon who had worked with *Brown* and *Conway*, sought a more flexible model than skin grafts for the study of allograft phenomena and eventually succeeded in obtaining functioning kidney transplants in dogs[16]. It was he who led the team which obtained the first clinical success with kidney transplantation between identical twins[17] and then with kidney allografts, using azathioprine for immuno-suppression[18,19]. Recently *Murray* received the Nobel prize 1990 for his achievements in medicine. His successes led to a dramatic surge for clinical accomplishments in organ transplants, and stimulated others to reinspect the work of *Hopfner* and *Carrel*.

In the 1960s, immunosuppression for prolongation of composite tissue allografts (CTAs) was achieved with methods impractible in man. *Schwind* used parabiosis before 2 weeks of age in rats to successfully transfer a limb for a 14-day period[20]. *Lapchinsky* achieved

immunological 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[21,22].

Once 6-mercaptopurine, azathioprine and prednisone had proven their beneficial effect in kidney transplantations[23] these immunosuppressive agents were also used in CTA transplantation. *Goldwyn et al* could only slightly prolong dog limb allograft survival with 6-mercaptopurine and azathioprine[24]. Rejection could not be prevented and in some cases fatal systemic drug-induced side effects occurred. Most interestingly, in this paper a cadaver human hand transplantation is reported to have taken place in 1964[25]. Complete allograft rejection occurred after 14 days already, despite cortisone and azathioprine therapy and local irradiation to the graft. At the time of their removal, the transplanted parts were too necrotic for microscopic assessment of the rejection process[26]. *Doi* encountered similar results in rats with a maximum of 24 days of CTA survival, suppressing the immune response by means of azathioprine and prednisone[27]. The only study showing prolonged survival before the introduction of Cyclosporine A (CyA) was that by *Lance et al*[28] who transplanted hind limbs between unrelated beagles using combinations of various potent ways to obtain immuno-suppression. The most successful results were obtained in three animals receiving a short-term course of massive immunosuppressive drug therapy, with or without splenectomy or thymectomy, followed by induction of immune tolerance from donor splenic cells or exchange transfusion. Despite a somewhat unstable course, long-term graft survival was achieved; one limb was rejected on day 200, and the other two survived beyond 60 and 300 days respectively.

The first data on survival of allogeneic limb transplants with CyA immunosuppression were published in the early 1980s by the group of *Furnas, Black* and *Hewitt*[27-31]. Lewis rats that received limbs from hybrid Brown Norway/Lewis rats treated with a 20 day course of 25 mg/kg CyA showed a dramatically increased transplant survival, with at that time at least one animal keeping its allogeneic limb beyond 225 days. *Fritz et al* could repeat these results and obtained indefinite CTA survival provided that the treatment with moderate doses of CyA was continuous[32]. Tolerance to limb allografts in rats could probably, partly or totally be attributed to the development of donor-host chimeras[33,34].

Recently orthopedic surgeon *Arai* demonstrated that the newly developed immunosup-pressive drug FK506 that is reported to be more potent than CyA (Chapter I, Section B.3.1.6), also significantly prolonged limb allograft survival in rats[35]. A 14-day FK506 treatment of 1 mg/kg yielded a mean graft survival time (MST) of 149.5 (SD=64) days and a single dose of 50 mg/kg even prolonged limb MST to 104.4 (SD=17) days.

However, for study into the feasibility of allogeneic hand transplantation in man, research

in rodents and dogs only yields limited information and results can not be extrapolated directly to the situation in evolutionary higher developed species. For immunological and phylogenetic reasons the nonhuman primate is obviously a more representative experimental animal, this was substantiated many years ago[36]. So far only two hand transplantation studies in the nonhuman primate (baboon) have been reported, respectively by the groups of *Daniel et al*[37] in 1986 and *Stark et al*[38] in 1987. In these two studies long term hand transplant survival occurred in two out of four (188 and 304 days) and one out of eight monkeys (296 days), respectively. In both studies high dosis of CyA and prednisone were used.

B.2 Aspects of allograft rejection

The labyrinthine interrelationship between the complex immunological cascade of cells and antibodies leading to rejection of a 'foreign' allograft is still not fully understood.

An immune response will be provoked when the major histo-compatibility complex (MHC) antigens between donor and recipient differ. As a result the allograft will be rejected unless the host immune response is modified by immunosuppression. The MHC in man, also known as the human leukocyte antigen (HLA) system, is an extremely complex genetic system. It includes the 'classical' gene loci which control the expression of MHC class I antigens: HLA-A, -B, -C, and MHC class II antigens: HLA-DR, -DQ, -DP. Each of these gene loci have multiple alleles resulting in an extensive polymorphism of the MHC[39-42]. Also a number of 'nonclassical' gene loci have been described for class I (HLA-E, -F, and -G) as well as for class II (HLA-DZα and -DOβ). Not all protein products of these nonclassical genes have been found or their physiological functions have yet to be established. In addition, there are several minor histocompatibility antigens which may also cause an early and irreversible rejection even in the presence of identical MHC[43], however in the rest of this section only the relevance of the 'classical' MHC antigens will be discussed.

Traditionally, MHC antigens from the donor organ are processed by antigen presenting cells (APC) in the recipient and are presented by these specialized cells (probably dendritic cells) to T-helper/inducer (Th/i) lymphocytes of the CD4+ phenotype[a], to initiate the immune response (Fig. 2). The CD4+ Th/i-cells recognize the foreign antigen in association with recipient MHC class II antigen on the presenting cell (this is known as MHC class II

---

[a]CD = Cluster of Differentiation, as defined during the Leucocyte Typing Conferences in Paris, 1982; Boston, 1984; Oxford, 1986; and Vienna, 1989.
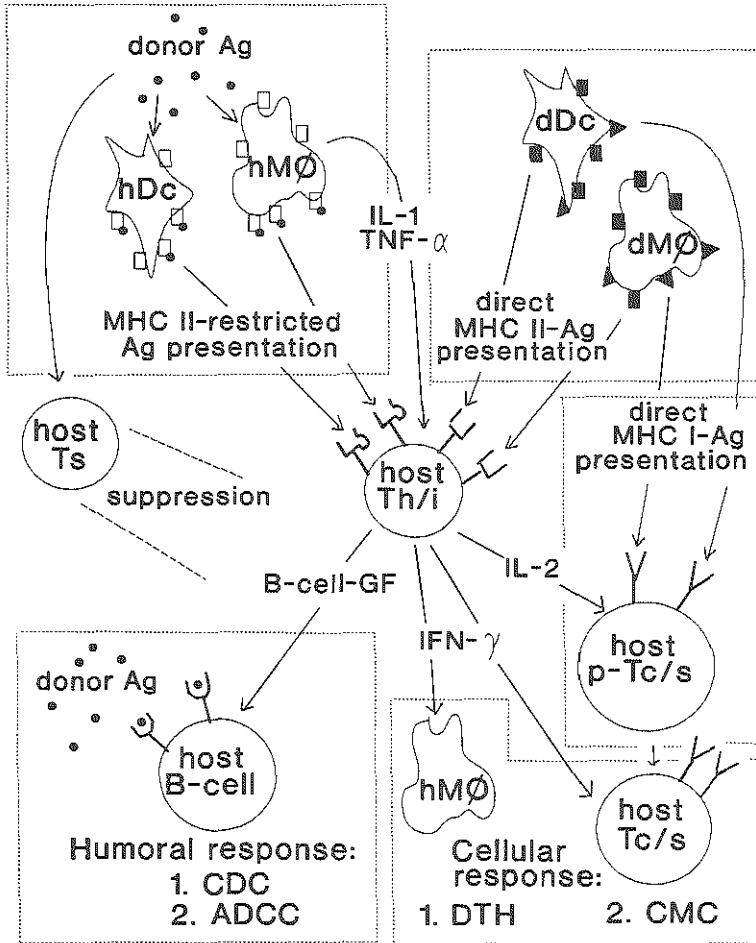
Figure 2. A simplified representation of the immune reponse against foreign tissue.
Abbreviations used: Ag=antigen; hDc=host dendritic cell; hMØ=host macrophage; dDc=donor dendritic cell; dMØ=donor macrophage; IL-1=interleukine 1; TNF-α=tumor necrosis factor-alpha; MHC I,II=major histocompatibility complex class I-, II-antigens; Ts=suppressor T-cell; Th/i=helper-inducer T-cell; IL-2=interleukine 2; B-cell-GF=B-cell growth factor; IFN-γ= interferon-gamma; p-Tc/s=precursor-cytotoxic-suppressor T-cell; Tc/s=cytotoxic-suppressor T-cell; CDC=complement dependent cytotoxicity; ADCC=antibody dependent cellular cytotoxicity; DTH=delayed type hypersensitivity; CMC=cell mediated cytotoxicty.

restriction). However, in the case of a vascularized graft, the APC will be of donor origin. It seems possible that recipient Th/i-lymphocytes entering the graft can recognize incompatible MHC class II antigen as presented by these specialized dendritic cells of the donor directly, without the need for processing by host presenting cells. Moreover, it is also possible that these donor dendritic cells leave the allograft and interact with recipient Th/i-cells at a more central site such as the spleen[44].

Once the recipient Th/i-cell has recognized the foreign antigen it produces a whole series of lymphokines resulting in a true amplification of the rejection response. Interleukine 2 (IL-2) is produced, and in concert with IL-1, a cytokine produced by antigen activated macrophages[45,46], also expression of IL-2 receptors (IL-2R) is increased[47,48]. IL-2R is not only expressed on Th/i-lymphocytes but also on some B-cells, macrophages, dendritic cells, and MHC class I restricted CD8+ T-cells or the T cytotoxic/suppressor lymphocyte subset (Tc/s)[49]. IL-2 reacting with these receptors will drive IL-2R-expressing antigen-activated cells to clonal expansion and will ensure viability. Also indirectly via stimulation of the release of B-cell growth factor, interleukines stimulate the proliferation of antigen activated B-cells to produce donor specific antibodies[50]. At the same time, the release of lymphokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) offer another way of amplifying the immune response. IFN-γ mediates the chemotactic migration and activation of T cells[51] and macrophages[52] and enhances the cytotoxic activity of natural killer cells, monocytes, cytotoxic T cells and polymorphonuclear leucocytes[53,54]. Like IFN-γ, TNF-α increases the expression of membrane glycoproteins such as intercellular adhesion molecule-1 (ICAM-1), relevant for the adhesion of circulating granulocytes, monocytes and lymphocytes to endothelial cells[55,56]. TNF-α also exerts direct cytotoxic effects towards endothelial cells[57]. Moreover, IFN-γ as well as TNF-α both enhance the expression of MHC class I and II antigens on almost every cell type[58-61]. Increased expression of MHC antigens of donor as well as host origin will initiate a vicious circle as the antigenicity of the allograft will be increased[62-64], MHC class II restricted activation of Th/i-cells will be increased[65,66] and subsequent induction of cell-mediated cytotoxicity will be enlarged[67].

The cellular reactions aimed at destroying an allograft, can be divided into a delayed-type hypersensitivity (DTH) and cell mediated cytotoxicity (CMC)[68-71]. In the effector phase, the DTH process is a non-specific reaction resulting in tissue destruction by monocytes, macrophages and polymononuclear leucocytes. During CMC, however, CD8+ Tc/s-lymphocytes will induce allogeneic cell lysis in a MHC class I restricted way. Both cellular reactions seem to take part in the rejection response of the host, CMC activity during the beginning of the effector phase of rejection, later phases of rejection may involve non-T killer activity as significant numbers of B lymphocytes, mononuclear cells, histiocytes and

natural killer (NK) cells invade the allograft also.

Humoral immunological reactions can damage a graft via complement dependent cytotoxicity (CDC) and via antibody dependent cellular cytotoxicity (ADCC)[68,69,72]. CDC implicates activation of the cascade of complement factors resulting in lysis of the cell to which these complement factors are bound via an immune complex, e.g. antibodies bound to the surface of an allografted cell. In ADCC, antibody-binding between a target cell specifically bound on one side to an effector cell and non-specifically bound on the other side of the antibody, results in lysis of the target cell by the effector cell also.

To produce and maintain an immunologic homeostasis in the host after 'successful' rejection or acceptance of an allograft, suppressor T (Ts) cells of the CD4+ as well as CD8+ phenotype play a pivotal role[73,74]. It has been demonstrated that in the induction phase of host unresponsiveness, Ts-cells in lymphoid tissue were antigen specific; however, those at the graft site gave a nonspecific suppressor effect[75]. CD4+ Ts-lymphocytes alone were responsible for induction of unresponsiveness but both CD4+ and CD8+ Ts-cells were operative in the maintenance phase[76,77]. However, it should be stressed that the relationship between phenotype and function is not absolute; the same cell may express different phenotypes under varying conditions of stimulation. There even is a vivid discussion about the true nature of suppressor cells and their suppressor function.

It is almost needless to emphasize that the immune response to an allograft is a very complex set of cellular and humoral interactions and that this section remains an incomplete synthesis of what is known about this subject, today.

B.3 Modification of the immune response

As clarified in the previous sections, allografts (except one from an identical twin) will be rejected by the host unless intervention can prevent rejection. Many non-specific and more specific methods of immunosuppression have been developed and tested as more organs are being transplanted. In this chapter, however, only the effect of matching for MHC antigens and those immunosuppressants that have been used in this research project will be discussed in detail (sections B.3.1.1-3.1.6). In this respect, a number of different immunosuppressive treatment schedules will be discussed (section B.3.2). Other ways to suppress the host immune response against foreign tissue will be reviewed briefly in section B.3.3.

B.3.1 Modification of the immune response relevant to this thesis

B.3.1.1 Matching for major histocompatibility antigens

Nowadays, there seems to be a fair consensus that matching for MHC class I and II antigens has a beneficial effect on organ allograft prognosis and patient survival rates[78]. However, there seems to be an organ related influence as to whether matching for MHC class I antigens is of more importance than matching for MHC class II-DR antigens. The effect of matching for MHC-DR antigens, is thought to outweigh matching for MHC class I antigens in kidney transplantation. In a prospective study in the rhesus monkey it was clearly demonstrated that mixed lymphocyte culture (MLC) nonresponsiveness (= MHC class II compatibility), had a profound effect on kidney graft prognosis, while matching for MHC class I antigens had no discriminating influence[79,80]. This could be confirmed in clinical kidney transplantation trials[81-83]. In skin transplantation studies in man, however, MLC reactive host/donor combinations matching for MHC class I-A and -B antigens correlated with improved graft survival times[84]. In the rhesus monkey it also led to a modest but distinct prolongation of skin graft survival times[85], whereas MLC nonresponsiveness between donor and host only had a minor beneficial effect, if any at all[86].

With the advent of cyclosporine A (CyA) there has been some question as to whether matching is still relevant. With regard to kidney transplants both the UCLA registry and the European Collaborative study with large numbers of patients still show a significant influence of matching for HLA-DR in patients treated with CyA[87,88]. Also a correlation between matching for MHC-DR and cardiac allograft survival under CyA treatment has been demonstrated[89].

B.3.1.2 Prednisolone

Since the early sixties prednisolone, the active compound of the glucocorticosteroid prednisone, has been successfully used as a conventional post-transplant immuno-suppressant[90]. Very few other drugs, with the exception of azathioprine and the alkylating agent cyclophosphamide, have been used as widely in clinical transplantation. The clinical effect of prednisolone may be due to a combination of its anti-inflammatory activities and its immunosuppressive properties[91-93]. The anti-inflammatory activities result in reduced migration of neutrophils in response to chemotactic stimuli as well as in reduced inflammation by inhibiting lysosomal enzyme release by neutrophils. The influence of prednisolone on the suppression of the immune response is complex and might result from a

combination of different immunosuppressive effects. Intra-nuclear activities, at the level of transcription have been demonstrated to result in modifications of enzyme synthesis (either induction or repression)[92,93]. Moreover, inhibition of the production of the cytokines IL-1, IL-2 and IFN-γ[92-96] will clearly have a immunosuppressive effect by hindering the cascade of the cellular and humoral immune response (section B.2). Furthermore, steroids are reported to interfere in the process of antigen presentation and recognition by their effect on MHC class II expression on antigen presenting cells[95,97].

B.3.1.3 Blood transfusions

Oplez et al were the first to demonstrate, in a retrospective analysis of clinical data, that nonspecific transfused kidney allograft recipients had superior graft survival as compared with nontransfused patients[98]. This finding was confirmed in experimental studies in rodents[99,100], dogs[101] and the rhesus monkey[102] as well as in clinical studies[103]. Since these reports in the early seventies many controversial results have been published with regard to the number of transfusions, the composition and preparation of the transfusate, the timing of the transfusions and the number of (mis)matched for MHC class I and/or II antigens that would yield best graft survival rates, it is still widely accepted that pretransplant blood transfusion to the recipient produces a favorable effect on graft survival. Even under CyA therapy third party blood transfusions have been demonstrated to have a beneficial effect on allograft survival times[104].

Theories dealing with the possible mechanism of the transfusion effect concern non-specific mechanisms (recipient selection[105], clonal deletion[106], iron overload and macrophage blocking[107]) humoral mechanisms (MLR-blocking antibodies[108], anti-idiotypic antibodies[109]) and cellular mechanisms (suppressor cell induction[110,111]).

B.3.1.4 Cyclosporine A

In 1972, Borel was the first to demonstrate the immunosuppressive activity of cyclosporine A (CyA), a fungal metabolite extracted from a fungus - *Tolypocladium inflatum Gams* - which was originally isolated from soil samples collected from the Hardanger Vidda in Norway[112]. CyA is a neutral, highly lipophilic, cyclic peptide containing 11 amino acids (Fig. 3). Though the exact mechanism of action of CyA is not known, its immunosuppressive activity is thought to be due to at least two properties: 1) its ability to

inhibit the production of lymphokines such as IL-2, IFN-γ, B cell differentiation factor and cytotoxic differentiation factor and 2) inhibition of the response to IL-2 of precursor cytotoxic effector lymphocytes[23,91,113]. At the same time, T-suppressor cell expression is not extensively disturbed[114,115]. On the molecular level CyA penetrates the nucleus of its target cell and interacts with sites on the chromosome, specifically disordering the transcription of messenger RNA coding for lymphokines. As a result, the synthesis and release of the above mentioned lymphokines is inhibited[116] and thus a more or less selective immunosuppression impairs the T-helper/inducer lymphocyte function. As can be seen from Figure 2 (section B.2), this will have enormous influence on the cascade of the rejection response. Importantly, CyA does not appear to directly affect granulocyte or macrophage function[117], and has hardly any myelosuppressive effect. Thus, CyA inflicts general host resistance less than more widely acting immunosuppressants such as azathioprine and prednisolone.

Cyclosporine A

Fig. 3. Structure of Cyclosporine A

CyA which came into generalized use in 1983, has proven to be the most successful primary drug in preventing rejection. It improved the 1-year graft survival from a previous 50-55% to over 80% in recipients of renal grafts from unmatched cadaveric donors[118]. With or without steroids 3-year kidney allograft survival could be prolonged to 70% to 80%, clearly better than could be obtained with azathioprine and steroids treatment[23,119].

Despite these positive properties, literature also mentions a number of major side effects of CyA. Most frequently reported are; moderate to severe nephrotoxicity, hepatotoxicity, tremors, hirsutism, gingival hypertrophy, central nervous system toxicity, and gastrointestinal intolerance. All side effects have shown to be dose-related and reversible on stopping the drug[23,120-122]. Soon after the introduction of CyA substantial concern arose that lymphomas might be more frequently observed after CyA treatment than with conventional immunosuppression[118]. Subsequent clinical studies could not confirm this[123] and neither could a recent study with data from over 4500 patients from 76 centers in 17 countries with patient follow-up over a 2- or 5-year period[124]. Initially CyA doses were much higher than currently recommended, which may, in part, explain the differences.
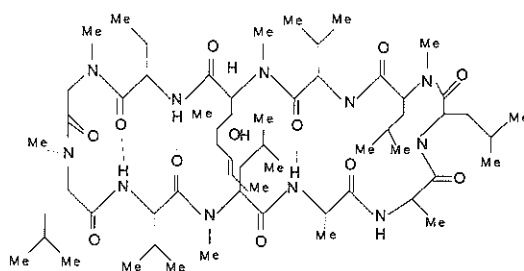
B.3.1.5 Monoclonal antibodies

Heterologous anti-lymphocyte serum (ALS) or anti-thymocyte globulins (ATG) have been used for years to treat or prevent rejection with considerable success[125]. But such heterologous sera (usually made in horses or rabbits) are very broad in their reactivity not only to leucocytes but often to other targets, such as platelets. Inevitably there is also a substantial batch-to-batch variability in efficacy and toxicity. Therefore, monoclonal antibodies (MAbs), which are by definition homologous in composition and monospecific, seem to be a more promising tool for the modulation of the immune response. Using the hybridoma technique of Köhler and Milstein[126], it became possible to develop reactive monospecific immunoglobulins (Fig. 4) with target structures that can vary from cell-surface-markers to free molecules (like cytokines).

The basic structure of Immunoglobulin   G
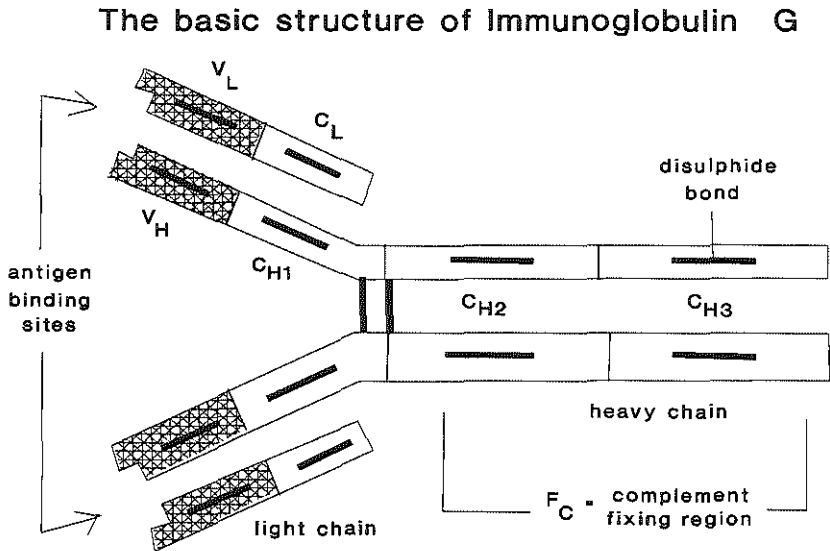
Figure 4. Structure of an immunoglobulin. Four polypeptide chains make up the immunoglobulin molecule. Sequence variability (V) occurs in the shaded areas in both the heavy (H) and light (L) chains, which are referred to as the V_H and V_L regions respectively. The rest of the molecule has a relative constant (C) structure. The constant portion of the light chain is termed the C_L region. The constant portion of the heavy chain is further divided into three structurally discrete regions: C_{H1}, C_{H2} and C_{H3}. These globular regions, which are stabilized by disulfide bonds, are referred to as domains. The sites at which the antibody binds antigen are located in the variable domains. Fc indicates the complement fixing region.

With such MAbs i.e. selective T cell populations relevant for rejection can be manipulated, without impairing the host's immune competence. Moreover, because of these properties, MAbs are most useful in several diagnostic tests (Chapter II, Section C).

Several mechanisms of action have been proposed to explain the immunosuppressive effect of MAbs: 1) MAbs can bind directly to antigen, thereby preventing a potential antigenic stimulus[127], 2) MAbs may inhibit adhesion processes involved in the immune response[128], 3) MAbs binding to cell-surface antigens may inhibit immune function, which is known as coating[129], 4) MAbs bound to a functional lymphocyte subset marker may induce cell clearance, possibly by complement dependent cell lysis or by destruction through the mononuclear phagocytic system, also in the presence of complement[130], 5) and finally the MAbs-target-complex may also be removed from the cell a phenomenon which is called surface antigenic modulation[131]. The kinetics of the latter three cell-related mechanisms is likely to depend on (any combination of) the affinity of the MAb with its target, the isotype of the MAb, and the nature and density of the target antigen (see also Chapter III, section D).

Clinical results with MAbs for the treatment of rejection episodes are mainly limited to the CD3 antigen. The first patient trial was performed by Cosimi et al, who used OKT3, which is CD3 specific, for the treatment of established kidney graft rejection episodes without other treatment and with concomitant lowering of steroid treatment. Within two to seven days in eight out of eight patients allograft rejection could be reversed[132]. Subsequent larger kidney transplant studies confirmed the beneficial effect of OKT3 and demonstrated that the efficacy of OKT3 (reversal rate 94%) was significantly better than that with conventional steroid treatment (reversal rate 75%)[133]. OKT3 has also been reported to be effective for the reversal of kidney and liver allograft rejection episodes that were nonreversible by high-dose steroids and/or ATG[134,135]. Clinical trials with other MAbs such as anti-T12 (CD6-specific[136]), RFT2 (CD7-specific[137]), WT32 (CD3-specific[138]), and CBL1 (blast-cell specific[139]) also showed reversal of acute renal allograft rejection, in 43%, 60%, 88%, and 89% of the cases, respectively.

Numerous experimental studies have been performed and still are being performed to test newly developed MAbs. Noteworthy are efforts to increase the specificity of the immunosup-pressive effect by trying to impair the function of subsets of T-cells. In this respect, MAbs specific for CD4+ T-cells have been tested and found to possess immunosuppressive potencies in transplant studies of different organs in the rodent[140,141] and non human primate[142,143]. Also some MAbs specific for CD8+ T-cells[143-145] and IL-2-receptor expressed by activated T-cells[146] appeared to suppress the immune response. Furthermore, new approaches in using MAbs for immunosuppression are being developed. Strom et al. replaced the DNA sequences of Diphteria toxin that code for the receptor-binding protein

domain with human IL-2 DNA sequences. In this way a recombinant IL-2 toxin hybrid molecule could be produced that showed immunosuppressive potencies[147].

Clearly, the potential for the use of MAbs in transplantation is enormous. Nevertheless, several side effects have been reported related to MAbs therapy. CD3- and CD8-specific MAbs may cause adverse reactions shortly after the first injection[148,149]. Fever, chills, bronchospasms and shock-like symptoms are most frequently observed and are explained by a massive release of lymphokines[133,148,149]. This problem might be circumvented by changing the Fc portion of the immunoglobulin from an IgG2a to an IgA variant, as the Fc portion of the CD3 MAb (IgG2a-isotype) determines the activation and subsequent lymphokine release of the target cell[150]. The major drawback of murine MAb therapy is the induction of an anti-mouse antibody (AMA) response, generally consisting of anti-isotype and anti-idiotype antibodies[133,145,148]. Obviously, such an AMA-response is unwanted because especially the anti-idiotype antibodies are found to abrogate therapeutical effectiveness of those injected MAbs they are specific for. Moreover, anti-isotype antibodies can cause an anaphylactic reaction when a second or third MAb treatment is given. To prevent sensitization against the murine immunoglobulin, 'idiotype switching' has been reported to be successful[151], though repeated courses of different MAbs may lead to the formation of crossreactive anti-idiotype antibodies causing unresponsiveness to a large number of MAbs[152]. High doses of concomitant immunosuppression at the time of MAb therapy will prevent an AMA-response[152,153], however this implies a possible intoxication of the MAb recipient. Theoretically, a human or humanized MAb would offer the best chances to prevent the occurrence of an anti-isotype response as their Fc tail is expected to be less immuno-geneic than the Fc tail of a murine MAb. Theoretically, this approach would also minimize the formation of neutralizing anti-idiotype antibodies. The first clinical trials with humanized MAbs do not permit any conclusions yet[154,155]. However, in a preclinical study in the chimpanzee, humanized anti-CD4 did not elicit a significant anti-anti-CD4 repsonse (M. Jonker, personal communications).

B.3.1.6 FK506

In 1987, a powerful new anti-T cell agent, designated FK-506, was first described[156,157]. The drug was extracted from the fermentation broth of the soil fungus *Streptomyces Tsukubaensis*. Although it is structurally distinct from CyA (Fig. 5) it possesses similar if not identical immunosuppressive properties and at the same time is approximately 100 times more potent than CyA in *in vitro* studies. Currently it is thought that the primary target of

FK-506, like that of CyA, may be a transcription activator(s) (or factors controlling its synthesis) required for lymphokine gene expression[158]. In this way, in vitro 'early' phase CD4+ T (helper/inducer) cell gene expression and secretion was blocked for IL-2, IL-3, IL-4, IFN-γ and TNF-α. Constitutively expressed genes (encoding i.e. MHC class I) and 'late' phase activation genes (encoding i.e. IL-2R, transferrin receptor or TNF-ß) were not affected by FK-506.

In vivo experiments revealed that at considerably lower doses than those of CyA (10-100 times less), allograft survival of rat hearts[159] as well as

FK-506

Fig. 5. Structure of FK-506

kidneys in beagle dogs could be prolonged[160]. Although in dogs major complications such as anorexia and vascular changes were observed[160-162], in other animals no such severe side effects occurred[162]. Subsequently in a clinical trial oral administration of FK-506 in low doses was demonstrated to be an effective and safe immunosuppressant for liver, kidney and pancreas transplantation[163]. FK-506 was less nephrotoxic than CyA and did not cause hypertension. However, many questions about its mode of action still need to be solved and longer experience of FK-506 in clinical trials is required before a fuller assessment of its relative merits, compared with CyA, can be made.
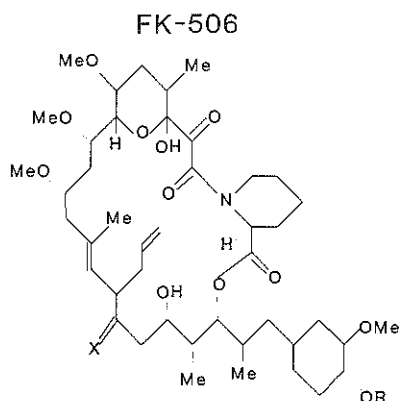
B.3.2 Treatment schedules

As most of the side effects of CyA therapy proved to be dose-dependent (Section B.3.1.4), attempts were made to devise protocols which used lower doses of CyA with or without the concomitant use of other drugs. In this respect, and to further increase graft and patient survival rates, a number of different approaches have been tested.

Steroids with CyA did not significantly improve kidney graft survival compared to CyA alone[164,165], though in comparison to conventional immunosuppressive therapy three-year graft and patient survival was better[119]. In one trial, data were presented, suggesting that the degree of nephrotoxicity was reduced with the addition of steroids[165].

Conversion some months after transplantation from CyA to azathioprine (AZA) and steroids lead to a dramatic improvement of renal function on cessation of CyA[166-169]. Provided

that there is an overlap of immunosuppressive therapies around the time of conversion, it would seem that high incidences of graft rejection within the first month of conversion could be prevented.

Double therapy with CyA and AZA did not improve graft survival rates, but showed less graft rejection compared to CyA monotherapy[170].

A controlled trial comparing triple therapy consisting of low doses of CyA, AZA and steroids with high-dose CyA and steroids showed no difference in graft survival times[171]. However, there were more rejection episodes in the triple therapy group and more nephrotoxicity and infection in the high-dose CyA group.

Quadruple therapy, consisting of addition of ALG for the first 14 days (later reduced to 7 days) to a triple therapy regimen also showed excellent graft survival times both for re-grafts as well as first grafts[172].

Sequential therapy by giving CyA after cessation of initial ALG therapy together with AZA and steroids, provided that renal function is adequate is also practiced by many transplantation centers[165,173]. The use of OKT3 instead of ALG in the same set-up would be accompanied with an unacceptable mortality and infection rate despite the occurrence of less rejection episodes[174]. Experience with prophylactic use of MAbs is still limited. Several other studies confirmed the lower incidence of rejection episodes during the first few weeks following transplantation[175,176]. Eventually however, graft survival rates and kidney function are often comparable to controls and hence at this moment the major therapeutic advances of MAb treatment appears to be at the time of allograft rejection.

The majority of the data presented in this section concern protocols for kidney transplantation. There are insufficient data concerning the use of these protocols in transplantation of other organs to be sure that the same conclusions apply, though it does seem likely[177].

B.3.3 Other ways of modifying the immune response

Many other ways of suppressing the host immune reponse against an allograft have been described and tested. In this section only a brief selection is presented.

Azathioprine (AZA), an agent which blocks DNA replication after it is metabolized *in vivo* to 6-mercaptopurine, has been mentioned earlier in this section. AZA was the first proven immunosuppressive reagent to be used clinically[18]. Originally it was thought to be a rather weak immunosuppressant, however, in combination with CyA and/or steroids (section B.3.2) it has clearly proven to be useful.

Total lymphoid irradiation (TLI) or ALG with or without subsequent bone marrow transplantation or low-dose immunosuppressants to induce long term donor specific unresponsiveness, is causing renewed interest[178,179].

Obviously, pharmaceutical industries try hard to develop completely new drugs to improve current immunosuppressive regimes for commercial reasons. In this respect FK506 which has been described in section B.3.1.6, can be mentioned. Structural similarities between FK506 and rapamycin (Fig. 6) has stimulated researchers to reinvestigate the immunosuppressive properties

Rapamycin

Fig. 6. Structure of Rapamycin

of rapamycin in a transplantation model[180]. Renal allograft survival in dogs and pigs could be prolonged. RS-61443, a morpholinoethyl ester of mycophenolic acid, is a completely new drug which also suppresses the immune response against a heart allograft in mice[181]. Allogeneic skin, heart, islet cells and kidney survival could be prolonged by 15-Deoxyspergualin[182] in rats. Furthermore, A 85 4777, a homopiperazine derivate could prolong skin allograft survival in rats also[183].

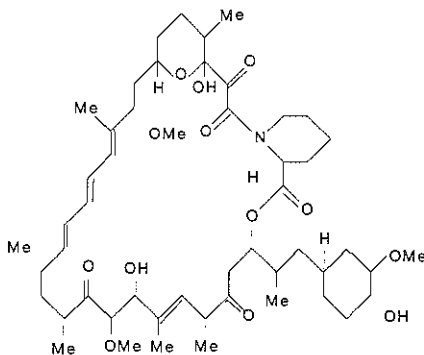Many more drugs could be mentioned and more will be developed in the coming years.

B.4 References

1. Bhishagratna KKL: An english translation of of the Sushrutta Samheta. 1907; vol. 1, Calcutta.
2. Tagliacozzi G: De Curtorum Chirurgia per Insitionem. Venice 1597.
3. Gnudi MT, et al: The sympathetic slave. In: The Life and Times of Gaspare Tagliacozzi. Los Angeles, Zeitlin & Ver Brugge 1976; pp285-286.
4. Hamilton D: In: Morris PJ. (Ed): Tissue Transplantation (Clinical Surgery International no. 3). Edinburgh, Churchill, Livingstone 1982; pp4-5.
5. Bünger C: Gelungener Versuch einer Nasenbildung aus einem völlig getrennten Hautstück aus dem Beine. Journal der Chirugie und Augenheilkunde 1823;4: 569.
6. Hopfner: Gefässtransplantation und Replantation amputirter Extremitäten. Verhandl. d. deutsch. Gesellsch. f. Chir., Berl. 1903; xxxii, pt.i, 45.
7. Carrel A: Results of the transplantation of blood vessles, organs and limbs, JAMA 1908; 51: 1626-1627.
8. Guthrie CC: Applications of blood vessel surgery. In: Blood Vessel Surgery. New York, Longman's, Green & Co 1912.
9. Padgett EC: Full-thickness skin graft in correction of soft tissue deformities. JAMA 1932; 98: 18.

10. Brown JB: Homografting of skin: with report of success in identical twins. Surgery 1937; 1: 558.
11. Gibson T, Medawar PB: Fate of skin homografts in man. J Anat 1943; 77: 299.
12. Converse JM, Duchet G; Successful homologeous skin grafting in war burn using identical twin as donor. Plast Reconstr Surg 1947; 2: 342.
13. Converse JM, Rapaport FT; Vascularization of skin autografts and homografts; experimental study in man. Ann Surg 1956; 143: 306.
14. Obituary. John Marquis Converse. Plast Reconstr Surg 1981; 68: 665.
15. Conway H, Sedar JD, Shannon JE Jr; Re-evaluation of the transparent chamber technique in the study of the circulation in autografts and homografts of skin. Transpl Bull 1957; 4(2): 62.
16. Murray JE, Lang S, Miller BF, Dammin GJ; Prolonged functional survival of renal autotransplantants in the dog. Surg Gynec Obstet 1956; 103: 15.
17. Murray JE, Merrill JP, Harrison JH: Renal homotransplantation in identical twins. Surg Forum 1956; 6: 432.
18. Murray JE, Merrill JP, Harrison JH: Prolonged survival of human kidney homografts by immunosuppressive drug therapy. N Engl J Med 1963; 268: 1315.
19. Murray JE, Tilney NL, Wilson RE: Renal transplantation: a twenty-five year experience. Ann Surg 1976; 184: 565.
20. Schwind JV: Homotransplantation of extremities of rats. Radiology 1962; 78: 806-809.
21. Lapchinsky AG: Recent results of experimental transplantation of preserved limbs and kidneys. Ann NY Acad Sci 1960; 87: 538.
22. Medical News-International Roundup. Tissue imbalance overcome in transplant of dog's leg. Med Tribune 1964; 5: 2.
23. Tilney NL, Strom TB: Chemical suppression of the immune responses. In: Progress in transplantation, volume 3. Morris PJ, Tilney NL (Eds), Chirchill Livingstone 1986; pp 1-31.
24. Goldwyn RM, Beach PM, Feldman D, Wilson RE: Canine limb homotransplantation. Plast Reconstr Surg 1966; 37: 184-195.
25. Medical Tribune and Medical News 1964; 5: 1.
26. Peter Bent Brigham Hospital Case No. 4-79-99.
27. Doi K: Homotransplanation of limbs in rats: a preliminary report on an experimental study with nonspecific immunosuppressive drugs. Plast Reconstr Surg 1979; 64: 613-624.
28. Lance EM, Inglis AE, Figarola F, Veith FJ: Transplantation of canine hind limb. J Bone Joint Surg (Am) 1971; 53: 1137-1149.
29. Black KS, Hewitt CW, Fraser LA, et al: Cosmas and Damian in the laboratory. N Eng J Med 1982; 306: 368-369.
30. Furnas DW, Black KS, Hewitt CW, et al: Indefinite composite tissue (limb) allograft survival in rats with cyclosporine : Possible furure implications in plastic and reconstructive surgery. Transpl Proc 1983; 15: 3063.
31. Hewitt CW, Black KS, Fraser LA, et al: Cyclosporine A (CyA) is superior to prior donor-specific blood (DSB) transfusion for the extensive prolongation of rat limb allograft survival. Transpl Proc 1983; 15: 514-517.
32. Fritz WD, Swartz WM, Rose S, Futrell JW, Klein E: Limb allografts in rats immunosuppressed with cyclosporine A. Ann Surg 1984; 199: 211.
33. Black KS, Hewitt CW, Fraser LA, et al: Composite tissue (limb) allografts in rats. II. Indefinite survival using low-dose cyclosporine. Transplantation 1985; 39: 365.
34. Hewitt CW, Black KS, Dowdy SF, et al: Composite tissue (limb) allografts in rats. III. Development of donor-host lymphoid chimeras in long-term survivors. Transplantation 1986; 41: 39.
35. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb

allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21: 3191.

36. Balner H. Dersjant H, Rood JJ van: A method to relate Leucocyte antigens and transplantation antigens in monkeys. Transplantation 1965; 3: 320.

37. Daniel RK, Egerszegi EP, Samulack DD, Skanes SE, Dykes RW, Rennie WRJ: Tissue transplants in primates for upper extremity
reconstruction: a preliminary report. J Hand Surg 1986; 11A:1.

38. Stark GB, Swartz WM, Narayanan K, Moller AR: Hand transplantation in baboons. Transplant Proc 1987; 19: 3968.

39. Auffray C, Strominger JL: Molecular genetics of the human major histocompatibility complex. Adv Hum Genet 1985; 15: 197-247.

40. Bjorkman PJ, Parham P: Structure, function, and diversity of class I major histocompatiblity complex molecules. Annu Rev Biochem 1990; 59: 253.

41. Kappes D, Strominger JL: Human class II major histocompatiblity complex genes and proteins. Annu Rev Biochem 1988; 57: 991.

42. Orr HT. In: Immunobiology of HLA. Ed B Dupont, 1989; 2:33. New York: Springer-Verlag.

43. Peugh WN, Superina RA, Wood KJ, Morris PJ: The role of H-2 and non-H-2 antigens and genes in the rejection of murine cardiac allografts. Immunogenetics 1986; 23: 30-37.

44. Larsen CP, Austyn JM, Morris PJ: The role of graft-derived dendritic leukocytes in the rejection of vascularized organ allografts. Recent findings on the migration and function of dendritic leukocytes after transplantation. Ann Surg 1990; 212: 308.

45. Newton RC: The production of human interleukin-1 beta by blood monocytes. Prog Clin Biol Res 1990; 349: 217.

46. Oppenheim JJ, Dubois CM, Ruscetti FW, Keller JR: Regulation of the production and receptors for interleukin-1, and tumor necrosis factor. Prog Clin Biol Res 1990; 349: 183.

47. Kuziel WA, Green WC: Interleukin-2 and the IL-2 receptor: new insights into the structure and function. J Invest Dermatol 1990; 94: 27S.

48. Smith KA: Interleukin 2 receptor. Annu Rev Cell Biol 1989; 5: 397.

49. Allouche M, Sahraoui Y, Augery-Bourget Y, Perrakis M, Jasmin C, Georgoulias V: Interleukin 2 receptors. Leuk Res 1990; 14: 699.

50. Inaba K, Granelli-Piperoni G, Steinmann RM: Dendritic cells induce T lymphocytes to release B cell stimulating factor by an interleukin 2 dependent mechanism. J Exp Med 1983; 157: 1339.

51. Issekutz TB, Stolz JM, Meide PH van der: Lymphocyte recruitment in delayed-type hypersensitivity: the role of IFN-g. J Immunol 1988; 140: 2989.

52. Nathan CF, Kaplan G, Levis WR, et al: Local and systemic effects of intradermal recombinant interferon-g in patients with lepromatous leprosy. N Engl J Med 1986; 315: 6.

53. Herberman RB. Ch. Interferon & cytotoxic effector cells in: Vilceck J & DeMaeyer E (Eds.) Interferon & the immune system. Amsterdam, Elsevier, 1984; 61.

54. Trinchieri G, Matsumoto-Kobayashi M, Clark SV, Sheera J, London L, Perussia B: Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 1984; 160: 1147.

55. Pober JS, Gimbrone MA, Lapierre LA, et al: Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. J Imunnol 1986; 137: 1893.

56. Leeuwenberg JFM, Jeunhomme TMAA, Buurman WA: Induction of an activation antigen on human endothelial cells in vitro. Eur J Immunol 1989; 19: 715.

57. Sato N, Goto T, Haranaka K. et al: Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition and cytotoxicity. JNCI 1986; 76: 1113.

58. Momburg F, Koch N, Moller P, Moldenhauer G, Hammerling GJ: In vivo induction of H-2K/D

antigens by recombinant interferon-γ. Eur J Immunol 1986; 16: 551.

59. Steiniger B, Meide PH van der, Westermann J, Klempnauer J: Systemic induction of class II MHC antigens after continuous intravenous infusion of recombinant gamma interferon in rats. Transpl Proc 1987; XIX (5): 4322.

60. Collins T, Lappiere LA, Fiers W, Strominger JL, Pober JS: Recombinant human tumor necrosis factor increases mRNA levels and surface expresion of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. PNAS 1985; 83: 446.

61. Leeuwenberg JFM, Damme J van, Meager T, Jeunhomme TMAA, Buurman WA: Effects of tumor necrosis factor on the interferon-g-induced histocompatibility complex class II antigen expression by human endothelial cells. Eur J Immunol 1988; 18: 1469.

62. Milton AD, Fabre JW: Massive induction of donor-type class I and class II major histocompatibility complex antigens in rejecting cardiac allografts in the rat. J Exp Med 1985; 61: 98.

63. Hävry P, von Willebrand E: The influence of the pattern of inflammation and administration of steroids on class II MHC antigen expression in renal transplants. Transplantation 1986; 42 (4): 358.

64. Ferry B, Halttunen J, Leszczynski D, Schellekens H, Meide PH van der, Hävry P: Impact of class II major histocompatibility complex antigen expression on the immunogenic potential of isolated rat vascular endothelial cells. Transplantation 1987; 44 (4): 499.

65. Pober JS, Gimbrone MA, Cotran RS, Reiss CS, Burakoff SJ, Fiers W, Ault KA: Ia expression by vascular endothelium is inducible by activated T cells and human gamma-interferon. J Exp Med 1983; 157: 1339.

66. Leszczynski L, Ferry B, Schellekens H, van der Meide P, Hävry P: Antagonistic effects of gamma-interferon and steroids on tissue antigenicity. J Exp Med 1986; 164: 1470.

67. Engleman EG, Benike JL, Grumet FC, Evans L: Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. J Immunol 1981; 127 (5): 2124.

68. Carpenter CB: Immunobiology of transplantation. In: Renal transplantataion. Garovoy MR and Guttmann RD (Eds), Churchill Livingstone 1986; pp 49-72.

69. Strom TB, Kupiec-Weglinksi JW, Tilney NL: On the mechanisms of rejection of vascularized organ allografts: a review and an attempt at synthesis. In: Progress in transplantation, volume 2. Morris PJ and Tilney NL (Eds), Churchill Livingstone 1985; pp126-146.

70. Hävry P: Intragraft events in allograft destruction. Transplantation 1984; 38: 1.

71. Intragraft rejection mechanisms. Immunol Rev 1984; 77.

72. Winn HJ: Antibody mediated rejection. In: Kidney transplantation, diagnosis and treatment. Williams GM, Burdick JF and Solez K (Eds), Marcel dekker inc. 1986; pp 17-27.

73. Hutchinson IV: suppressor T cells in allogeneic models. Transplantation 1986; 41: 547-555.

74. Dorf ME, Benaceraff B: Suppressor cells and immunoregulation. Ann Rev Immunol 1984; 2: 127.

75. Duncan WR, Bitter-Suermann H, Stepkowski SM: Induction of transplantation tolerance in rats by spleen allografts. III. The role of T suppressor cells in the induction of specific unresponsiveness. Transplantation 1987; 44: 553.

76. Padberg WM, Kupiec-Weglinski JW, Lord RH, et al; W3/25+ T cells mediate the induction of immunologic unresponsiveness in enhanced rat recipients of cardiac allografts. J Immunol 1987; 138: 3669.

77. Padberg WM, Lord RH, Kupiec-Weglinski JW, et al; Two phenotypically distinct populations of T cells have suppressor capabilities simultaneously in the maintanance phase of immunological enhancement. J Immunol 1987; 139: 1751.

78. Hors J, Busson M, Bouteiller AM, Raffoux C, Betuel H, Fauchet R, Tongio MM, Mercier P, Lepetit JC, Dausset J: Dissection of the respective importance of HLA-A,B,DR matching in 3,789

prospective kidney transplants. Transpl Proc 1987; 19: 687.

79. van Es AA, Marquet RL, van Vreeswijk W, Tank B, Balner H: Influence of matching for RhLA (SD) antigens, and of mixed lymphocyte reaction reactivity on allograft survival in unrelated monkeys. Transpl Proc 1977; 9: 257.

80. van Es AA, Balner H: Serological matching for D locus antigens improves kidney allograft survival in rhesus monkeys. Transplantation 1978; 26: 187.

81. Ting A, Morris PJ: Powerful effect of HLA-Dr matching on survival of cadaveric renal allografts. Lancet 1980; 2: 282.

82. Goeken NE, Thompson JS, Corry RJ: A 2-year trial of prospective HLA-Dr matchning. Transplantation 1981; 32: 522.

83. van Rood JJ, Persijn GG, Paul LC, Cohen B, Lansbergen Q, Goulmy E, Claas FHJ, Baldwin W, van Es LA: Significant factors influencing kidney graft survival. Transpl Proc 1981; 13: 909.

84. Koch CT, Frederiks E, Eijsvogel VP, van Rood JJ: Mixed lymphocyte culture and skin graft data in unrelated HLA identical individuals. Lancet 1971; ii: 1334.

85. Balner H, Dersjant H, van Vreeswijk W, Gabb BW: Tissue typing of rhesus monkeys: application in transplantation research. Transplant Proc 1972; 4: 3.

86. van Vreeswijk W, Balner H: The major histocompatibility complex, matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 1980; 30: 196.

87. Opelz G; Effect of matching in 10,000 cyclosporine-treated cadaver kidney transplants. Transpl Proc 1987; 19: 641.

88. Cicciarelli J, Terasaki PI, Mickey MR; The effect of zero HLA class I and II mismatching in cyclosporine-treated kidney transplant patients. Tranplantation 1987; 43: 636.

89. Yacoub M, Festenstein H, Doyle P, et al: The influence of HLA matchingin cardiac allograft recipients receiving cyclosporine and azathioprine. Transpl Proc 1987; 19: 2487.

90. Goodwin WE, Kaufman JJ, Mims MM: Human renal transplantation. I. Clinical experiences with sic cases of renal homotransplantation. J Urol 1963; 89: 13.

91. Bach JF, Strom TB: The mode of action of immunosuppressive agents. In: Research monographs in immunology 1985; volume 9: Elsevier.

92. Cupps TR, Fauci AS: Corticosteroid-mediated immunoregulation in man. Immunol Rev 1982; 65: 133.

93. Dupont E, Wybran J, Toussaint C: Glucocorticosteroids and organ transplantation. Transplantation 1984; 37: 331.

94. Dupont E, Huygen K, Schandené L, Vandercruys M, Palfiet K, Wybran J: Influence of in vivo immunosuppressive drugs on production of lymphokines. Transplantation 1985; 39: 143.

95. Snyder DS, Unanue ER: Corticisteroids inhibit murine macrophage Ia expression and interleukin 1 production . J Immunol 1982; 129: 1803.

96. Arya SK, Wong-Staal F, Gallo RC: Dexamethasone-mediated inhibition of human T cell growth factor and gamma-interferon mRNA. J Immunol 1984; 133: 273.

97. Warren MK, Vogel SN: Opposing effects of glucocorticosteroids on interferon-gamma induced murine macrophage Fc receptor and Ia antigen expression. J Immunol 1985; 134: 2462.

98. Opelz G, Sengar DP, Mickey MR, Terasaki PI: Effect of blood transfusions on subsequent kidney transplants. Transpl Proc 1973; 5: 253.

99. Marquet RL, Heystek GA, Tinbergen WJ: Specific inhibition of organ alllograft rejection by donor blood. Transpl Proc 1971; 3: 708.

100. Fabre JW, Morris PJ: The effect of donor strain blood pretreatment on renal allograft rejection in rats. Transplantation 1972; 14: 608.

101. Obertop H, Jeekel J, Vriesendorp HM, McDicken I, Westbroek DL: The effect of donor blood on renal allograft survival in DL-A tissue typed beagle littermates. Transplantation 1975; 20:49.

102. van Es AA, Marquet RL, van Rood JJ, Kalff MW, Balner H: Blood transfusions induce prolonged allograft survival in rhesus monkeys. Lancet 1977; i: 193.

103. Persijn GG: HLA-matching and blood transfusion(s) in renal transplantation. The Hague: Pasmans; 1985.

104. Hunsicker LG, Schanbacher BA, Goeken NE, Corry RJ: A randomized comparison of preoperative and intraoperative transfusions in recipients of first cadaveric renal allografts. Transpl Proc 1988; XX (1,S1): 286.

105. Salvatierra Jr O, Vincenti F, Amend W, etal: Deliberate donor-specific blood transfusions to living realted renal transplantation. A new appraoch. Ann Surg 1980; 192: 543.

106. Terasaki PI: The beneficial transfusion effect on kidney graft survival attributed to clonal deletion. Transplantation 1984; 37: 119.

107. Dupont E, Vereerstraeten P, Espinosa O, Tielemans C, Dhaene M, Wybran J: Multiple transfusions and T cell subsets: a role for ferritin. Transplantation 1983; 35: 508.

108. Petranyi GG, Padanyi A, Horuzsko A, Rethy M, Gyodi E, Perner F: Mixed lymphocyte culture-evidence that pretransplant transfusion with platelets induces FcR and blocking antibody antibody production similar to that induced by leukocyte transfusion. Transplantation 1988; 45: 823.

109. Reed E, Hardy M, Benevenisty A, et al: Effect of antiidiotypic antibodies to HLA on graft survival in renal allograft recipients. N Engl J Med 1987; 316: 1450.

110. van Rood JJ, Balner H: Blood transfusion and transplantation. Transplantation 1978; 26: 275.

111. Lagaaij EL, Ruigrok M, Braun LA. Blood transfusions induced changes in cell mediated lympholysis. Submitted for publication.

112. Borel JF: Editorial: Ciclosporin and its future. Prog Allergy 1986; 38: 9-18.

113. Hess AD, Colombani PM: Mechanism of action: in vitro studies. Prog Allergy 1986; 38: 198-221.

114. Hutchinson IF, Shadur CA, Duarte JSA, et al: Cycloporine A spares selectively lymphocytes with donor-specific suppressor characteristics. Transplantation 1981; 32: 210.

115. Hess AD, Colombani PM, Esa A. Cyclosporine: Immunobiologic aspects in transplantation. In: Kideny transplant rejection. Williams GM, Burdick JF, Solez K (Eds). Marcel Dekker inc 1986; pp353-382.

116. Borel JF, Ryffel B: The mechanism of action of ciclosporin: a continuing puzzle. In: Schindler R (Ed) Ciclosporin in autoimmune diseases. Berlin, Springer-Verlag, 1986; pp24-32.

117. Borel JF, Feurer C, Mangnee C, Stahelin H: Effects of the new anti-lymphocyte peptide cyclosporin A in animals. Immunology 1977; 32: 1017.

118. Calne RY, White DJG, Evans DB, et al: Cyclosporin A in cadaveric organ transplantation. Br Med J 1981; 282: 934.

119. The Canadian Multicenter Transplant Group. A randomized clinical trial of cyclosporine in cadaveric renal transplantation: Analysis at 3 years. New Engl J Med 1986; 314: 1219.

120. Kahan BD: Cyclosporine: Biological activity and clinical applications. Grune and Stratton 1984.

121. Wilczek H, Ringden O, Tyden G: Cyclosporine-associated central nervous system toxicity after renal transplantation. Transplantation 1985; 39: 110.

122. Keown PA, Stiller CR, Wallace AC. Nephrotoxicity of cyclosporine A. In: Kidney transplant rejection. Williams GM, Burdick JF, Solez K (Eds). Marcel Dekker inc 1986; pp423-458.

123. Penn I: Lymphomas complicating organ transplantation. Transpl Proc 1983; 15: 2790.

124. Cockburn I: Assessment of the risks of malignancy and lymphomas developing in patients using sandimmune. Transpl proc 1987; 19: 1804.

125. Jaffers GJ, Cosimi AB: Antilymphocyte globulin and monoclonal antibodies. In: Morris PJ (Ed)

Kidney Transplantation: Principles and Practice. 2nd edit. New York, London: Grune & Stratton 1984; 281-299.

126. Köhler G, Milstein C: Continous cultures of fused cells secreting antibody of defined specificity. Nature 1975; 256: 495.

127. Price RW: Herpes Zoster: An approach to systemic therapy. Med Clin North Am 1982; 66: 1105.

128. Krensky AM, Sachez-Madrid F, Robbins E, Nagy J, Springer TA, Burakoff SJ: The functional significane, distribution and structire of LFA-1, LFA-2 and LFA-3: cell surface antigens involved with the CTL-target interaction. J Immunol 1983; 131: 611.

129. Burakoff SJ, Weinberger O, Krensky AM, Reiss CS: A molecular analysis of the cytolytic T lymphocyte response. Adv Immunol 1984; 36: 45.

130. Bach JF, Dardenne M, Chatenoud L: Immunomodulation of T cells by thymic hormone and monoclonal anti-T cell antibody. Transpl Proc 1982; 14: 509.

131. Chatenoud L, Baudrihaye MF, Kreis H, Goldsteinb G, Schindler J, Bach JF: Human in vivo antigenic modulation induced by the anti-T cell OKT3 monoclonal antibody. Eur J Immunol 1982; 12: 979.

132. Cosimi AB, Burton RC, Colvin RB, Goldstein G, Delmonico FL. LaQuaglia P, Tolkoff-Rubin N, Rubin RH, Herrin JT, Russell PS: Treatment of acute renal allograft rejection with OKT3 antibody. Transplantation 1981; 32: 535.

133. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. New Eng J Med 1985; 313 (6): 337.

134. Ponticelli C, Rivolta E, Tarantino A, Egidi F, Banfi G, DeVecchi A, Montagnino G, Vegeto A: Clinical experience with ORTHOCLONE OKT3 in renal transplantation. Transpl Proc 1986: 18: 942.

135. Starzl TE, Fung JJ. ORTHOCLONE OKT3 in treatment of allografts rejected under Cyclosporine-steroid therapy. Transpl Proc 1986; 18: 937.

136. Milford EL, Carpenter CB, Kirkman RL, Tilney NL, Mazoujian G, Strom TB, Lazarus JM, Schlossman SF, Guttmann RD, Lowry R, Rocher L, Campell Jr DA, Salomon DR, Pfaff WW: Anti-T12 monoclonal antibody therapy of acute renal allograft rejection. Transpl Proc 1987; 19: 1910.

137. Raferty MJ, Lang CJ, Ivory K, Tidman N, Sweny P, Fernando ON, Moorhead JF, Janossy G: Use of RFT2 (CD7) monoclonal antibody as prophylaxis against renal allograft rejection. Transpl Proc 1985; 17: 2737.

138. Tax WJM, van de Heijden HMW, willems HW, Hoitsma AJ, Berden JHM, Koene PJA: Immunosuppression with monoclonal anti-T3 antibody (WT32) in renal transplantation. Transpl Proc 1987; 19: 1905.

139. Takahashi H, Okazaki H, Terasaki PI, Iwaki Y, Kinukawa T, Taguchi Y, Chia D, Hardiwidjaja S, Miura K, Ishizaka M, Billing R: Reversal of transplant rejection by monocloanl antiblast antibody. Lancet 1983; ii: 1155.

140. Cobbold SP, Waldmann H: Skin allograft rejection by L3T4 and Lyt-2+ T cell subsets. Transplantation 1986; 41: 634-639.

141. Madsen J, Peugh WN, Wood KJ, Morris PJ: The effect of anti-L3T4 monoclonal antibody treatment on first-set rejection of mouse cardiac allografts. Transplantation 1987; 44(6): 849.

142. Cosimi AB, Burton RC, King PC, et al: Evaluation in primate renal allograft recipients of monoclonal antibody to human T-cell subclasses. Transpl Proc 1981; 13: 499.

143. Jonker M, Nooij FJM, Chatenoud L: Immunosuppression by monoclonal T cell antibodies. Transpl Proc 1986; 18: 1287.

144. Cosimi AB: Anti-T-cell monoclonal antibodies in transplantation therapy. Transpl proc 1983; 15: 1889.

145. Jonker M, Nooij FJM, van Suylichem P, Neuhaus P, Goldstein G: The influence of OKT8F treatment on allograft survival in rhesus monkeys. Transplantation 1986; 41: 431.

146. Diamantstein T, Osawa H, Kirkman RL, Shapiro ME, Strom TB, Tilney NL, Kupiec-Weglinski JW: Interleukin 2 receptor - a target for immunosuppressive therapy. In: Morris PJ, Tilney NL (Eds). Transplantation Reviews. New York: Grune & Stratton, 1987: 1: 177.

147. Strom TB. New strategies for immunosuppression. Presented at the XII International Congress of the Transplantation Society, 1988, Sydney.

148. Jonker M, Nooij FJM, den Butter G, Lambalgen R, Fucello AJ: Side effects and immunogenicity of murine lymphocyte specific monoclonal antibodies in sub-human primates. Transplantation 1988; 45: 677-682.

149. Chatenoud L, Ferrar C, Reuter A, Legendre C, Gevaert Y, Kreis H, Franchimont P, Bach JF: Systemic reaction to the anti-T-cell monoclonal antibody OKT3 in relation to serum levels of tumor necrosis factor and interferon-alpha. N Eng J Med 1989; 320: 1420.

150. Parlevliet KJ, Jonker M, ten Berge RJM, van Lier RAW, Wilmink JM, Sterngers PFW, Aarden LA, Schellekens PTA: Anti-CD3 murine monoclonal isotype switch variants tested for toxicity and immunologic monitoring in four chimpanzees. Transplantation (in press).

151. Jonker M, den Brok JHAM, Idiotype switching of CD4-specific monoclonal antibodies can prolong the therapeutic effectiveness in spite of host anti-mouse IgG antibodies. Eur J Immunol 1987; 17: 1547-1553.

152. Hricik PR, Zarconi J, Schulak JA: Influence of low dose cyclosporine on the outcome of treatment with OKT3 for acute renal allograft survival. Transplantation 1989; 47: 272-278.

153. Schroeder TJ, First MR, Mansour ME, Hurtubise PE, Hariharan S, Ryckman FC, Munda R, Melvin DB, Penn I, Ballistreri WF, Alexander JW: Anti-murine antibody formation following OKT3 therapy. Transplantation 1990; 49: 48-51.

154. Hamblin TJ, Catta AR, Glennie MJ: Initial experience in treating human lymphoma with chimeric univalent derivate of monoclonal anti-idiotype antibody. Blood 1987; 69: 790-797.

155. Hale G, Clark MR, Marcus R, Winter G, Dyer MJS, Philips JM, Reichmann L, Waldmann H: Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody Campath-1H. Lancet 1988; ii: 1399.

156. Kino T, Hatanaka H, Hashimoto M, et al: FK-506, a novel immunosuppressant isolated from the a Streptomyces. I. Fermentation, isolation and physio-chemical and biological characteristics. J Antibiot (Tokyo) 1987; 40: 1249.

157. Kino T, Hatanaka H, Miyata S, et al: FK-506, a novel immunosuppressant isolated from the a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. J Antibiot (Tokyo) 1987; 40: 1256.

158. Tocci MJ, Matkovich DA, Collier KA, et al: FK-506 selectively inhibits expression of early T cell activation genes. J Immunol 1989; 143: 718.

159. Ochiai T, Nakajima K, Nagata M, et al: Studies of the induction and maintenance of long-term graft acceptance by treatment with FK-506 in heterotopic cardiac allotransplantation in rats. Transplantation 1987; 44: 734.

160. Ochiai T, Nagata M, Nakajima K, et al: Studies of the effects of FK-506 on renal allografting in the beagle dog. Transplantation 1987; 44: 729.

161. Collier DS, Thiru S, Calne R: Kidney transplantation in the dog receiving FK-506. Transpl Proc 1987; 19: 62 (suppl 6).

162. Todo S, Ueda Y, Demetris JA, et al: Immunosuppression of canine, monkey, and baboon allografts by FK-506: With special reference to synergism with other drugs and tolerance induction. Surgery 1988; 104: 239.

163. Starzl TE, Todo S, Fung J, et al: FK-506 for liver, kidney and pancreas transplantation. Lancet

1989; 2: 1000.
164. Starzl TE, Weil R, Iwatsuki S, et al: The use of cyclosporine A and prednisone in cadaver kidney transplantation. Surg Gynecol Obstet 1980; 151: 17.
165. In: Morris PJ (Ed) Kidney Transplantation: Principles and Practice. 3rd edit. Philadelphia, WB Saunders 1988; p285.
166. Morris PJ, French M, Ting A, at al. In: Cyclosporine A. White D (Ed) Amsterdam, Elsevier 1982; p355.
167. Morris PJ, Chapman JR, Allen RD, et al: Cyclosporin conversion versus conventional immunosuppression: long-term follow-up and histological evaluation. Lancet 1987; 1; 586.
168. Hoitsma AJ, Wetzels JF, van Lier HJ, Berden JH, Koene RA. Cyclosporin treatment with conversion after three months versus conventional immunosuppression in renal allogarft recipients. Lancet 1987; 1: 584.
169. Hall BM, Tiller DJ, Hardie I, Hahony J, Mathew T, Thatcher G, Miach P, Thomson N, Sheil AGR: Comparison of three immunosuppressive regims in cadaver renal transplantation: long-term cyclosporine, short-term cyclosporin followed by azathioprine and prednisolone, and azathioprine and prednisolone without cyclosporine. N Engl J Med 1988; 318: 1499.
170. Salaman J: Cyclosporine moo-drug therapy. Transpl proc 1988; 20(Suppl 3): 117.
171. Ponticelli C, Taranto A, Montagnino, et al: A randomized trial comparing triple-drug therapy and double-drug therapy in renal transplantation. Transplantation 1988; 45: 913.
172. Simmons RL, Canafax DM, Fry DS, et al: New immunosuppressive drug combinations for mismatched related and cadaveric renal transplantation. Transpl Proc 1986; 18(Suppl 1): 76.
173. Henry ML, Bowers VD, Sommer BG, et al: Transpl Reviews. Morris PJ, Tilney NL (Eds), New York, Grune & Stratton 1988; 2: 55.
174. reported at 1988, Clinical Immunology and Transplantation Course, Lyon.
175. Khawand N, Ali A, Aquino A, Brems W, Light JA: A comparison of Minnesota antilymphocyte globulin (MAG) and OKT3 as initial therapy following cadaveric kidney transplantation. Transpl Proc 1988/1989 Sydney Tr Soc.
176. Norman DJ, Shield CF, Barry J, et al: Early use of OKT3 monoclonal antibody in renal transplantation to prevent rejection. Am J kidney Dis 1988; 11: 107.
177. Morris PJ: Single or multiple drug therapy. Transpl Proc 1989; 21: 820.
178. Sampson D, Levin BS, Hoppe RT, Rieber CP, Miller E, Waer M, Kaplan HS, Collins G, Strober S: Preliminary observations on the use of total lymphoid irradiation, rabbit antithymocyte globulin, and low-dose prednisone in human cadaver renal transplantation. Transpl Proc 1985; 17: 1299.
179. Monaco AP, Wood ML, Maki T, Madras PM, Sahyoun AL, Simpson MA: Attempt to induce unresponsiveness to human renal allografts with antilymphocyte globulin and donor-specific bone marrow. Transpl Proc 1985; 17: 1312.
180. Calne RY, Collier DStJ, Lim S, et al: Rapamycin for immunosuppression in organ transplantation. Lancet 1989; 2: 227.
181. Morris RE, Hoyt EG, Murphy MP, Eugui EM, Allison AC; Mycophenolic acid morpholinoethylester (RS-61443) is a new immunosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. Transpl Proc 1990; 22: 1659.
182. Dickneite G, Schorlemmer HU, Walter P, Thies J, Sedlacek HH; The influence of (+/-)-15-deoxyspergualin on experimental transplantation and its immunopharmaceutical mode of action. Behring Inst Mitt 1986; 8: 93.
183. Schorlemmer H, Bartlett RR, Wolf E, Seiler FR; Prologation of allogeneic transplanted skin grafts by A85 4777, a new immunosuppressive homopiperazine derivate. Transpl Proc 1990; 22: 1671.

CHAPTER I: INTRODUCTION
SECTION C: Rationale of the experiments

S.E.R. Hovius & H.P.J.D. Stevens

C.1 Rationale of the experiments
C.1.1 Objectives & experimental animals

Interest in the use of allogeneic composite tissue to reconstruct congenital or acquired deformities of extremities is centuries old (Chapter I, Section B). In the Netherlands, there is clinical demand from patients (and/or their parents) for reconstruction of large extremity defects that can not be solved by autogenous tissue transfer (Chapter I, Section A). Especially with the advent of Cyclosporine A (CyA), monoclonal antibodies (MAbs) and the possibility of microsurgical operations, a new stimulus has been given to this field of composite tissue transplantation.

Several authors have provided data on the feasibility of composite tissue allograft (CTA) transplantation under various kinds of immunosuppression, but mainly in a rat hind limb transplantation model[1-9]. Rodent models for tissue transplantation give leads as to what technical, functional and immunological aspects are of importance. In this respect, our group performed a limb transplantation study in the rat to monitor the onset of rejection. However, the limitations of studies in less developed animals are clear with regard to the preclinical testing of the afore mentioned aspects of CTA-transplantation. Only in a higher developed species such as the nonhuman primate, can more substantial extrapolations to the situation in man be made with regard to the technical feasibility of CTA-transplantation. Only then can it be tested optimally whether or not functional recovery can be expected and what immunological events occur during the immune response of the host and therapeutic intervention to suppress this response. The rhesus monkey was chosen as an experimental animal. Research was performed at the Primate Center TNO as in this institute ample experience existed with transplantation studies in these animals. Conventional immunosuppressants and polyclonal anti-lymphocyte sera were tested *in vivo* in the late sixties[10,11] and seventies[12] already. Also anti-human MAb have been successfully applied *in vivo* to modify the immune response[13,14]. Non-human primates are more likely to fulfill the requirements for testing the efficacy and safety of immunosuppressive drugs and MAbs in particular[14,15]. Furthermore, the (immunological) status of the rhesus monkeys is well defined. All monkeys are serologically typed for a large number of MHC antigens, which show close resemblance with the MHC antigens of man[16-18]. Recently, identification of new MHC alleles is being established by restriction fragment length polymorphisms (RFLP) analysis also[19].

Some of the topics of interest have been elucidated in two hand transplantation studies in the baboon[21,22]. However, even high doses of CyA in combination with prednisolone could not prevent rejection of the allogeneic hand transplant in the majority of the cases, nor could an increase in steroid treatment reverse all rejection episodes effectively. Moreover,

functional recovery testing was not performed longitudinally.

It was therefore our objective to evaluate the technical feasibility of an allogeneic transplantation model of the radial side of the hand in the rhesus monkey with special emphasis on improving the necessary immunosuppressive regime. Longitudinal monitoring was performed with respect to allograft survival, sensory and motor function recovery, and several immunological parameters both in the peripheral blood of the host as well as in the allograft itself. Also, the immunosuppressive effect was tested of several monoclonal antibodies and a new immunosuppressive drug 'FK-506' in a skin transplantation model in rhesus monkey and rat, respectively.

C.1.2 Ethical aspects

Initially it was the intention to transplant the complete hand in a rhesus monkey model, for the ultimate goal was to explore the possibility of allogeneic transplantation of the complete hand in patients who lack one or both hands due to congenital deformity or trauma. However, during discussions with members of the ethical committee of the Primate Center TNO, Rijswijk it was agreed to transplant the radial side of the hand only. In this way, both allograft donors and recipients would still maintain fairly good function of the hand if the first ray of the hand had to be amputated. Loss of the radial side of the hand (the thumb) does interfere with pinch and key grip but not with grasp and support function of the affected hand. From a research point of view, such a complete functional unit as a transplant of the radial side of the hand would still allow for all possible technical, functional and immunological investigations.

Indeed, post-experiment evaluation revealed that 13 out of 14 allograft donors could use their operated hands for almost normal grasp and support functions. Also all allograft recipients that survived the research project had good grasp and support function of the operated hand after amputation of a completely rejected allograft.

All experiments were performed in consultation with the veterinary physician of the Primate Center and the Article 14 Officer of the Government, who makes sure that all regulations regarding animal research are followed.

C.1.3 Studies performed

In section A of chapter I, the historical background of hand reconstruction and the development of microsurgery is reviewed. The inability to reconstruct a patient with an amputated hand has been the objective for our reserach model. The set-up for this model from a technical and functional point of view is described. In section B, historical and immunobiological aspects of composite tissue allografting are presented. More detailed information is given with regard to the immunosuppressive drugs that have been used in this thesis. In section C, the rationale of this research project are given together with some ethical considerations.

In chapter II, five introductory studies are described dealing with A) the value of different factors for the monitoring of the rejection of an allogeneic hind limb in the rat; B) the anatomy of the upper extremity of the rhesus monkey and the partial hand we transplanted in particular; C) the experiences that were encountered using a thermoplastic splint for the fixation of the upper extremity in the rhesus monkey; D) the value of several anti-lymphocyte MAbs for the suppression of the immune response and the effect of combining seven of these MAbs; and E) the crossreactivity of a panel of anti-human MAbs with rhesus surface markers in immunohistochemical analysis on frozen sections.

With the knowledge gained from the studies described in chapter II, 12 consecutive allogeneic transplantations of the radial side of the hand were performed successfully in the rhesus monkey. In chapter III, study A) deals with the technical aspects of these operations; study B) describes the results from longitudinal tests into the recovery of sensory and motor functional recovery of the transplant; and study C) deals with the lymphocyte subset fluctuations that were observed in the peripheral blood as well as in the allograft itself. Furthermore, in study D) the encountered complications were investigated with regard to correlation with historic and therapeutic factors. The occurrence of four post-operative lymphoprolifertave disorders under immunosuppression and their association with Simian T-Leukemia retrovirus infections of the graft recipients is described in study E.

Finally, in chapter IV two new approaches to improve immunosuppressive therapy were investigated in skin transplantation studies. Study A deals with the synergistic effect of the combination of two MAbs specific for two potent cytokines in the rhesus monkey. Study B describes the beneficial effect of the new drug FK-506 in combination with CyA, in the rat.

In chapter V and hoofdstuk VI an overall discussion and summary is presented in English and Dutch, respectively.

C.2 References

1. Schwind JV: Homotransplantation of extremities of rats. Radiology 1962; 78: 806-809.
2. Doi K: Homotransplantation of limbs in rats: a preliminary report on an experimental study with nonspecific immunosuppressive drugs. Plast Reconstr Surg 1979; 64: 613-624.
3. Black KS, Hewitt CW, Fraser LA, et al: Cosmas and Damian in the laboratory. N Eng J Med 1982; 306: 368-369.
4. Furnas DW, Black KS, Hewitt CW, et al: Indefinite composite tissue (limb) allograft survival in rats with cyclosporine. Transpl Proc 1983; 15: 3063.
5. Hewitt CW, Black KS, Fraser LA, et al: Cyclosporine A (CyA) is superior to prior donor-specific blood (DSB) transfusion for the extensive prolongation of rat limb allograft survival. Transpl Proc 1983; 15: 514-517.
6. Fritz WD, Swartz WM, Rose S, Futrell JW, Klein E: Limb allografts in rats immunosuppressed with cyclosporine A. Ann Surg 1984; 199: 211.
7. Black KS, Hewitt CW, Fraser LA, et al: Composite tissue (limb) allografts in rats. II. Indefinite survival using low-dose cyclosporine. Transplantation 1985; 39: 365.
8. Hewitt CW, Black KS, Dowdy SF, et al: Composite tissue (limb) allografts in rats. III. Development of donor-host lymphoid chimeras in long-term survivors. Transplantation 1986; 41: 39.
9. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21: 3191.
10. Balner H, Dersjant H: Effects of antilymphocyte sera in primates. In: Antilymphocytic serum (Eds Wolstenholme GEW and O'Conner M) CIBA Foundation Study Group No 29, Little, Brown and Co, New York, 1967; p85.
11. Balner H, Eysvoogel VP, Cleton FJ: Testing of anti-human lymphocyte sera in chimpanzees and lower primates. Lancet 1968; ii: 19.
12. van Es AA, Balner H: The influence of blood transfusions and matching for DR antigens on kidney allograft survival in unrelated rhesus monkeys. Transplantation 1979; 28: 2.
13. Jonker M, Goldstein G, Balner H: Effects of in vivo administration of monoclonal antibodies specific for human T cell subpopulations on the immune system in a rhesus monkey model. Transplantation 1983; 35: 521.
14. Jonker M, Neuhaus P, Zurcher C, Fucello A, Goldstein G: OKT4 and OKT4A antibody treatment as immunosuppression for kidney transplantation in rhesus monkeys. Transplantation 1985; 39: 247.
15. Jonker M: The importance of non-human primates for preclinical testing of immunosuppressive monoclonal antibodies. Seminars in Immunology, 1990, (in press).
16. Balner H, Dersjant H, van Rood JJ: A method to relate leukocyte antigens and transplantation antigens in monkeys. Transplantation 1965; 3: 230.
17. Balner H, van Vreeswijk W: The major histocompatibility complex of rhesus monkeys: a short review. J Med Primatol 1975; 4: 382.
18. van Vreeswijk W, Roger JH, D'Amaro J, Balner H; The major histocompatibility complex of rhesus monkeys RhLA-A. VII. Tissue Antigens 1977; 9: 17.
19. Bontrop RE, Otting N, Broos LA, Noort MC, Kenter M, Jonker M; RFLP analysis of the HLA-, ChLA-, and RhLA-DQ alpha chain gene regions: conservation of restriction sites during evolution. Immunogenetics 1989; 30(6): 432.
20. Daniel RK, Egerszegi EP, Samulack DD, Skanes SE, Dykes RW, Rennie WRJ: Tissue transplants in primates for upper extremity reconstruction: a preliminary report. J Hand Surg 1986; 11A:1.
21. Stark GB, Swartz WM, Narayanan K, Moller AR: Hand transplantation in baboons. Transplant Proc 1987; 19: 3968.

CHAPTER II: INTRODUCTORY STUDIES

STUDY A   : Postoperative monitoring of allogeneic limb transplantation in rats

S.E.R. Hovius

A.1 Introduction

Allogeneic limb transplantations have been performed in rats before[3,4,7]. Without immunosuppressive drugs the life expectancy of a transplanted limb in general does not exceed three weeks. With the well-known, strong immunosuppressive drug cyclosporine A transplanted limbs remained vital for as long as this drug has been given. Due to the side-effects of immunosuppressive drugs it is important to keep the dosage as low as possible without losing the allogeneic transplant. Information about the initial onset of rejection is therefore essential. The earlier the onset of rejection is detected the earlier a change in the immunosuppressive drug scheme can be initiated and thereby prevent possible severe damage to vital tissues in the transplant[2]. The purpose of this study is to find parameters which indicate the initial onset of rejection.

Clinical examination is not always easy to interpret and when skin changes are evident rejection is already advanced. Frequent histologic examination is in practice difficult. In the clinical patient we have gained experience with postoperative monitoring after microvascular surgery, especially temperature, transcutaneous oxygen and laser doppler flowmetry[9]. In the allogeneic limb transplantation model in the rat, temperature and transcutaneous oxygen measurements were not used by us, because temperature measurements are unreliable and easily influenced[1,6] and the transcutaneous oxygen probe is to large to measure on a rat limb transplant. Therefore we decided to use peripheral blood gases instead. In rejection, a decline in glucose and increase of lactate peripheral blood levels could be expected due to the hypoxic state of the transplant[8,10]. These were also included in the study.

Laser doppler flowmetry (LDF) was used to assess flux in the microcirculation of the skin. LDF has proven to be valuable as described by others[5,11].

Since we were not able to obtain blood samples and histology from the transplanted limb repeatedly, all measurements were done in every rat only once on the random day of sacrifice. A pilot study had been performed to obtain technical experience with the model. In this pilot study none of the allogeneic limb transplantations survived two weeks, therefore we did all our experiments between days 2 and 14 after transplantation.

A.2 Materials and methods

A.2.1 Experimental design

Allogeneic limb transplantations were performed on 20 rats. Inbred specific pathogen-free BN/Bi rats were used as donors and Wag/Rij rats were used as acceptors. This combination represents a very strong mismatch. In the control group 6 limb transplantations were done on Wag/Rij rats. Weights of the rats ranged between 315-400 grams. They were not used for other experiments.

The planned day of post mortem study was determined for each rat in a random fashion, with the restriction of an even distribution of the rats over the relevant period. In the allogeneic group all experiments were done between days 2 and 14 and in the control group between days 7 and 304. On the day of obduction all animals were subsequently subjected to: clinical examination, laser doppler flowmetry (LDF), bloodgases, glucose, lactate and biopsies for histological examination in the transplanted limb as well as in the contralateral limb.

In clinical examination the transplant was always compared to the normal contralateral limb. The following parameters were scored: edema, colour, hairloss, epidermolysis, crust formation and exsudation. LDF measurements (Perimed PF 2) were taken in all animals during 5 minutes on the same three locations, i.e. distal and proximal volar aspect as well as the dorsum of the foot. The obtained graphic representation was divided into 5 blocks of 1 minute. The median of each block was assessed from the values taken at 6, 18, 30, 42 and 54 seconds. The medians of each block were combined to achieve a mean value for every location. We used the mean value of the three locations to obtain an overall mean value as a representative LDF measurement for every limb.

Duplicate peripheral bloodgases, glucose and lactate were taken from the toes of the transplant and the contralateral limb after intravenous injection of 1 ml heparine solution (50 IU/ml). Bloodgases were measured with the AVL 945 automatic blood gas system. Glucose was tested with the glucoquant (Boehringer), lactate with the lactate UV-test (Boehringer). The mean of the obtained duplicate values was considered as representative.

Histological examination was done from the skin, the subcutaneous soft tissue and the bone at the height of the proximal foot. Histological grading consisted of none; epidermal changes and inflammatory infiltration; mononuclear infiltration; obstructive vascular changes and diffuse mononuclear infiltration; hemorrhage and necrosis. Bone marrow rejection was scored as present or not. All gradings were done by the same pathologist in one session.

A.2.2 Surgical technique

We used the same procedure as described by Fritz et al. in 1984[3]. After induction and maintenance of anaesthesia with an ether gauze the vessels of the donor limb were mobilized through an inguinal approach. The femoral vessels were dissected inferiorly in continuity with the saphenous vessels which run superficially along the medial aspect of the leg.
All other branches, including the profunda femoris and the popliteal vessels were ligated with bipolar coagulation. The saphenous vessels were dissected free to the level of the midcalf, where amputation was performed leaving the leg attached to the long vascular pedicle. 1 ml Heparine solution (50 IU/ml) was given intravenously. At this point the recipient rat was prepared by mobilizing the femoral vessels to the level of the superfical epigastric vessels. A subcutaneous tunnel was formed by blunt dissection from the inguinal region to the mid dorsum. A 2 cm dorsal midline incision was made at the end of the subcutaneous tunnel. The donor and recipient femoral vessels were then clamped and divided. The limb was pulled through the subcutaneous tunnel with the toes first. Care was taken not to twist the long vascular pedicle. The limb was sutured to the dorsum of the recipient rat. After anastomosis of the femoral veins and arteries with 10-0 nylon, under the operating microscope, the clamps were released and blood flow observed.
A few drops of xylocaïn 2% were applied on the anastomoses. The operation was completed by the closure of the inguinal incision.
At the end of the operation the recipient rat was given 2 ml 0,9% NaCl intravenously as infusion and recovered under heatlamps at 37°C. Time of ischemia was 45-60 minutes. Total operation time was 2-3 hours.

A.2.3 Statistical analysis

For all parameters their measured values were related to time elapse. The degree of histologic rejection was compared to LDF values. The ratio of values measured simultaneously in transplanted and contralateral limb was taken as outcome in order to minimize surrounding influences (for example room temperature, depth of anesthesia etc.). For statistical analysis the rank correlation test according to Spearman was used. This test is known to be hardly influenced by extreme values. We used the one sided test because the direction of the correlation under the alternative hypothesis was known a priori.

A.3 Results

A.3.1 General results

In the study vascularised transplants were performed in 34 animals. 8 Animals were excluded from the experiment,i.e. 2 rats were lost during anaesthesia, 5 transplants were lost within 48 hours due to vascular thrombosis, 1 rat in the allogeneic group was excluded on day 12 following severe rejection and loss of the transplant.

In the control group (6 animals) limbs were transplanted from one Wag/Rij rat to another inbred Wag/Rij rat (Fig. 1). The transplanted limbs showed moderate edema, which resolved within a week. The limbs appeared healthy for as long as 10 months after operation, except for the toes which were nibbled of the denervated limb after two to three months postoperatively. In the allogeneic group (20 animals) limbs were transplanted from BN/Bi rats to Wag/Rij rats. The transplanted limbs showed progressive edema. Colour changes were difficult to assess due to brown donor limbs. Hairloss was seen in 5 rats after the 10th postoperative day. Epidermolysis, crust formation, exudation and leathery skin were progressively seen from the seventh postoperative day onwards.
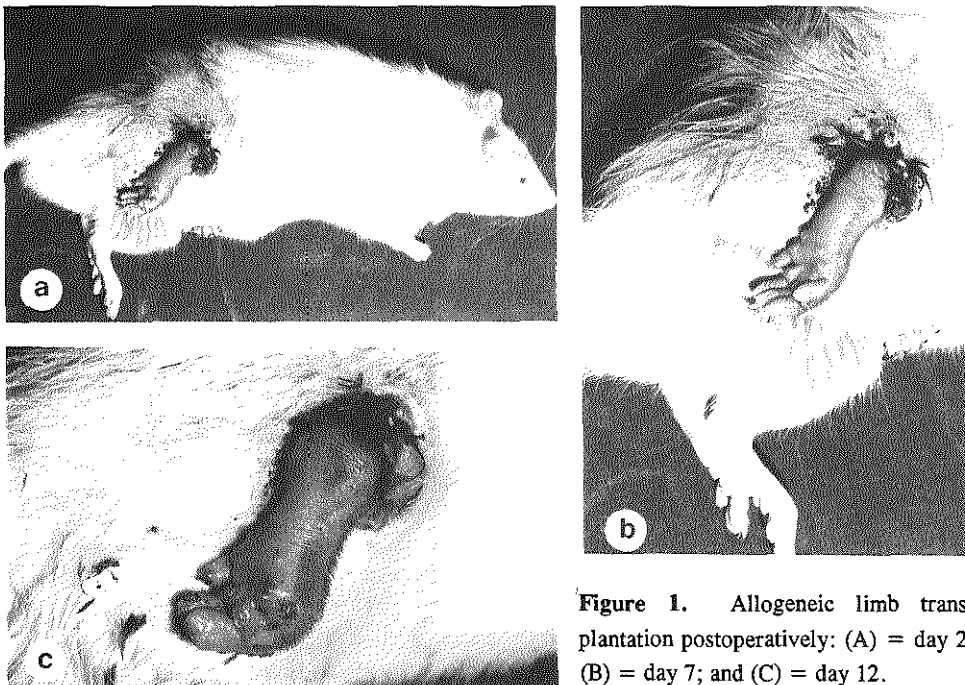

Figure 1. Allogeneic limb transplantation postoperatively: (A) = day 2; (B) = day 7; and (C) = day 12.

Peripheral blood gases from the toes of the transplanted limb and the contralateral limb taken on the planned day of obduction were valid in 13 rats. In 4 rats bloodgases were excluded due to a different sampling technique. Peripheral glucose and lactate were measured in respectively 10 and 8 rats, because after completion of blood gas sampling it was difficult to get enough blood for further examination (Table I). Peripheral blood gases, glucose and lactate were statistically not significant in relation to time elapse.

In 16 rats of the allogeneic group a reliable graphic representation was obtained. This was also achieved in all rats of the control group (6 rats). In the allogeneic group the LDF measurements were not statistically significant for the contralateral own limb in relation to time elapse ($p > 0,05$). This of course is as expected, the variation in absolute values however are substantial. The LDF measurements for the transplanted limb and the ratio of transplanted and contralateral limb were statistically significant in relation to time ($p < 0.001$ and $p < 0.0001$ respectively) (Fig 2).

A.3.2 Histology

The earliest morphological changes in the allotransplanted limbs become manifest on day 4 after surgery (Fig 3a).
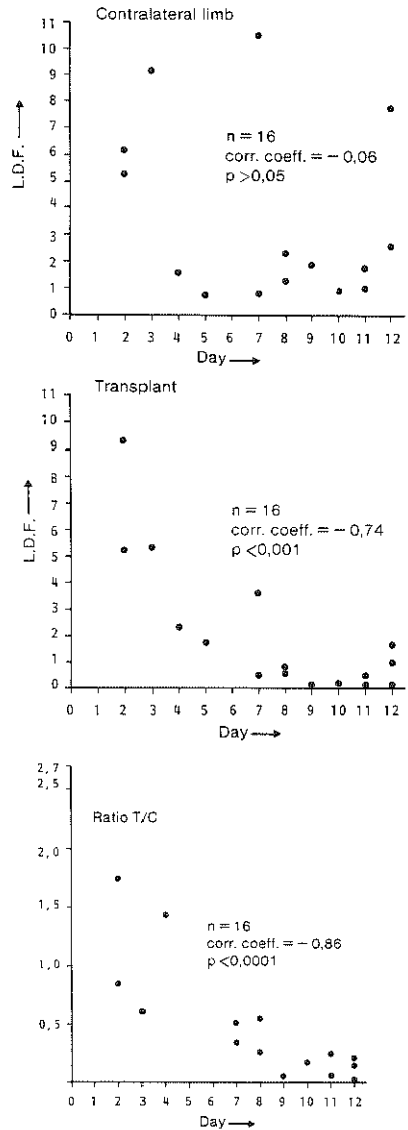

Figure 2. Laser Doppler flowmetry (LDF) in the allogeneic group related to time. (Ratio T/C= ratio of transplanted limb and contralateral limb).

Table 1. Measured factors in allogeneic limb transplantations in rats related to time.

Parameter	Contralateral limb			Transplant			Ratio T/C		
	n	corr. coëff.	P	n	corr. coëff.	P	n	corr. coëff.	P
pO_2	13	-0,05	>0,05	13	0,15	>0,05	13	0,26	>0,05
pCO_2	13	0,24	>0,05	13	0,17	>0,05	13	-0,07	>0,05
pH	13	-0,02	>0,05	13	-0,19	>0,05	13	-0,33	>0,05
Lactate	8	-0,51	>0,05	8	-0,64	>0,05	8	-0,16	>0,05
Glucose	10	-0,13	>0,05	10	-0,31	>0,05	10	0,01	>0,05
Laser Doppler Flowmetry	16	-0,06	>0,05	16	-0,74	<0,001	16	-0,86	<0,0001

According to Spearman's Rank Correlation Test
Correlation coëfficient and one-sided p-value corrected for ties
The same Parameters in Isologeous Rats were not significant (p>0,05)
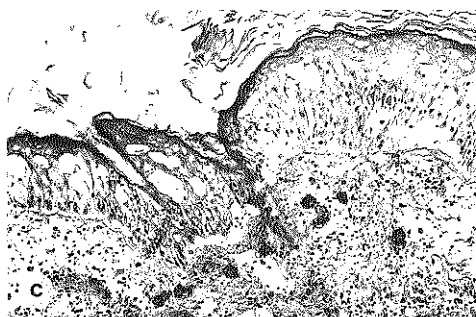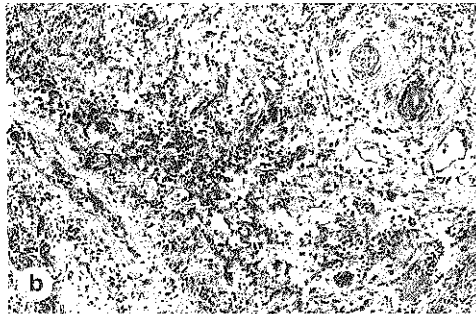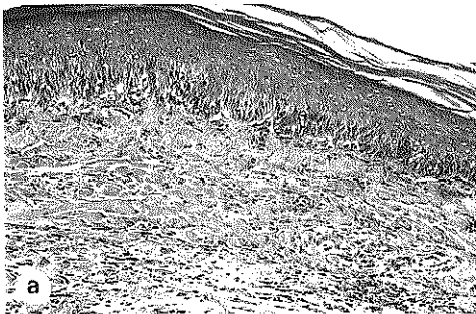

Figure 3a. Day 4. The epidermis shows a slight damage with spongiosis, nuclear pycnosis and lymphocytic infiltration. In the upper parts of the reticular dermis there is a mild mononuclear inflammatory infiltration. (H&E, original magnification x 180).

Figure 3b. Day 8. A dense diffusely distributed inflammatory infiltration in the dermis. (H&E, original magnification x 180).

Figure 3c. Day 12. Severe epidermal changes consisting of spongiosis, cell vacuolation and nuclear pycnosis. The papillary dermis shows severe diffuse edema, inflammatory infiltration and occlusion of the capillary blood vessels (H&E, original magnification x240).

The changes consisted of focal areas of moderate epidermal spongiosis and dyskeratosis, they were accompanied by a polymorphous leucocytic inflammatory infiltration of the papillary and the reticular dermis. The capillary blood vessels were strikingly dilated and filled with red blood cells. At the fifth and the seventh day a mononuclear inflammatory infiltration was distinctly observed in the dermal layers and in the subcutis. The infiltration had a patchy distribution mostly arround the capillaries and the small venules. The cell population was mostly lymphocytic, but some large mononuclear cells with vesicular nuclei and eosinophylic cytoplasma were seen.

The infiltration becomes more dense and was diffusely distributed on day 8 (Fig. 3b). Occlusive lesions of the dermal capillaries and small sized vessels, accompanied by endothelial damage and followed by desintegration of the vessel wall were the most prominent vascular alterations at that time. Frequent invasion of the periostium and the bone marrow was apparent.

The progressive obliterations of the dermal vessels resulted in ischemic necrosis of the transplants. It was evident by days 12 to 14 (Fig. 3c). The rejection of the transplanted limb for soft tissue and bone marrow in relation to time was significant ($p=0.02$). This was not the case for periosteal rejection ($p=0.10$). In relation to the LDF measurements soft tissue rejection was significant for the transplanted limb ($p<0.05$) as well as for the ratio transplanted and contralateral limb ($p<0.01$; Fig. 4).

A.4 Discussion

Clinical examination of the allogeneic transplanted limb correlated well with histologic signs of rejection as has been often described[3,4,7]. After crust formation clinical examination is difficult, however if severe skin changes were seen in the presence of edema the underlying tissue was still viable. This was proven by blood loss after extraction of a toe nail. When this edema disappeared it meant loss of viability of the limb. Epidermolysis as a strong clinical evidence of rejection was seen from the 7th postoperative day onwards. Daniel and his group could reverse severe skin changes together with edema by changing the immunosuppressive treatment[2].

Peripheral blood gases as well as glucose and lactate were not useful as a parameter of rejection in our model.

LDF measurements showed a continuous decline after operation.
From the 7th postoperative day the values were consistently low (at most 55% of the contralateral limb). Before the 6th postoperative day all values were above 55% of the

contralateral limb. According to these values there is a significant correlation with clinical and histological rejection, as such the LDF is a good parameter. Early detection of transplant rejection is of great importance because a change of immuno-suppressive drugs dosage and/or scheme can still be administered to save the transplant.

In our model however LDF measure-ments could not be used as a predictor for the initial onset of rejection, because a clear turning point before the 7th postoperative day could not be detected.

In search for this turning point 7 rats were followed nearly daily with LDF measure-ments. Due to a great variety of values before the 7th postoperative day this point could not be produced. To reduce the great variety of values and to produce a turning point continuous measurements are preferred, as we are accustomed to do in the clinical patient. In the rat model this is not possible.

In conclusion, in the allogeneic limb transplantation model in the rat clinical and histologic examination as well as laser doppler flow measurements (LDF, $p < 0.0001$) are good parameters for rejec-

Figure 4. Rejection correlated to laser Doppler flow (LDF) in the transplanted limb. (Grading of soft tissue rejection: $0 =$ no rejection; $1 =$ epidermal changes and inflammatory infiltration; $2 =$ mononuclear infiltration; $3 =$ obstructive vascular changes and diffuse mononuclear infiltration; $4 =$ hemorrhage and necrosis. T/C = ratio of transplanted limb and contralateral limb).

tion. In contrast peripheral bloodgases, glucose and lactate were not useful as parameters for rejection. In this model however LDF values cannot predict an initial onset of the rejection before clinical signs are evident, presumably due to the impossibility of continuous measurements.
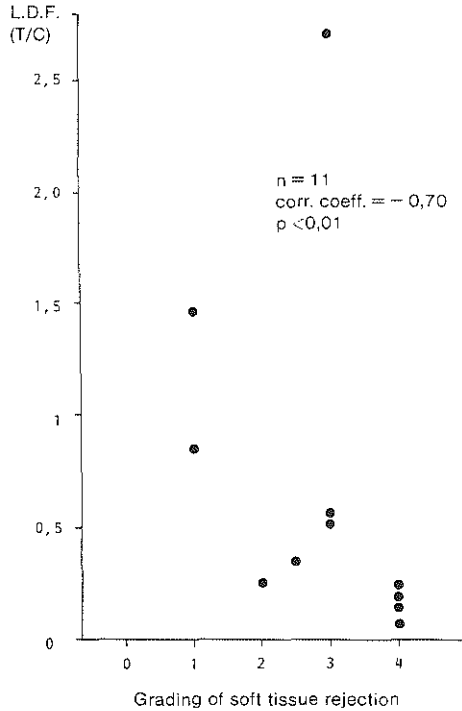
A.5 References

1. Black KS, Hewitt CW, Howard EB et al.: Diagnosis of rejection and functional analysis of composite tissue (CT) and skin allografts prolonged with cyclosporine. Transpl Proc 15: 3069, 1983.
2. Daniel RK, Egerszegi EP, Samulack DD et al.: Tissue transplants in primates for upper extremity reconstruction: a preliminary report. J Hand Surg [Am] 11: 1, 1986.
3. Fritz WD, Swartz WM, Rose S et al.: Limb allografts in rats immunosuppressed with Cyclosporin A. Ann Surg 199: 211, 1984.
4. Furnas DW, Black KS, Hewitt CW et al.: Cyclosporine and long-term survival of composite tissue allografts (limb transplants) in rats (with historical notes on the role of plastic surgeons in allotransplantation). Transpl Proc 15: 3063, 1983.
5. Heden PG, Hamilton R, Arnander C: Laser Doppler surveillance of the circulation of free flaps and replanted digits. Microsurgery 6: 11, 1985.
6. Jones BM: Monitors for the cutaneous microcirculation. Plast Reconstr Surg 73: 843, 1984.
7. Kim SK, Aziz S, Oyer P et al.: Use of cyclosporin A in allotransplantation of rat limbs. Ann Plast Surg 12: 249, 1984.
8. Peacock EE: Inflammation and the cellular response to injury. Wound Repair, 3rd edition, Philadelphia, Saunders, 1984.
9. Smith AR, Sonneveld GJ, Kort WJ et al.: Clinical application of transcutaneous oxygen measurements in replantation surgery and free tissue transfer. J Hand Surg 8: 139, 1983.
10. Smith AR, Kort WJ, van der Meulen JC: O_2 and glucose in gereplanteerde vingers en ledematen, een nieuwe bepaling voor de prognose. Ned Tijdschr Geneeskd 125: 1991, 1981.
11. Svensson H, Svedman P: Continuous monitoring of the circulation in flaps. VIII international congres of plastic surgery Jun 26 - Jul 1, 1983.

CHAPTER II: INTRODUCTORY STUDIES

STUDY B : Replantation of the radial side of the hand in the
rhesus monkey, anatomical and functional aspects of
a research model

S.E.R. Hovius & H.P.J.D. Stevens

B.1 Introduction

Clinical organ transplantation is successful[1, 2, 3], it is therefore logical to extend this field with experimental composite tissue allografts, as a preliminary to human hand transplantation. Experimental limb transplantations in the rat have been performed by many different groups[4, 5, 6, 7]. Prolonged survival of these limb allografts treated with the immunosuppressive agent Cyclosporine A was established. If however human hand transplantation is considered, functional studies should be performed on higher species, as for instance the non-human primate. The possibility of extrapolation to the human situation can thus be improved. Research was performed on the hand of the rhesus monkey in the early sixties. Replantations of thumb and index were caried out in those days to develop microvascular surgery[8]. In the baboon, hand transplantation has been performed by Daniel et al in 1986 and Stark et al in 1987[9, 10]. Long term allograft survival was established in two out of four and one out of eight monkeys, respectively. In these studies evidence of sensory and motor function recovery has been demonstrated.

The hand of the non-human primate is closely related to the human hand, regarding anatomy and functional use. For ethical and functional reasons transplantation of the whole hand was not performed in our studies. Instead the first ray enlarged with the radial forearm flap, was used as a model, still enabling research on sensory and motor function recovery. Basic handfunction in the remaining wrist and four fingers of the donor monkey in case of transplantation, could thus be maintained.

This paper is comprised of three parts. Firstly an anatomical study was done to define the radial unit, secondly four replantations of the radial side of the hand of rhesus monkeys were performed to establish the feasibility of the model and finally functional recovery was tested.

B.2   Materials and methods
B.2.1 Anatomical dissection

In fifteen fresh cadaver upper extremities of the rhesus monkey (Maccaca mulatta) the thumb and radial forearm flap were dissected to evaluate the muscles in the forearm and hand. In eight arms special attention was directed to the distal radial artery and cephalic vein, to the distal median, ulnar and superficial radial nerve as well as to the thenar musculature and distal part of the tendons around the first ray. The size of the radial forearm flap was set at about 20-25 cm^2.

B.2.2 Replantations of the radial side of the hand

To prove the technical feasibility of the research model, four replantations of the first ray of the hand with the radial forearm flap were performed, with a maximum follow-up period of three years.

B.2.2.1 Animals

Rhesus monkeys were born and raised in the TNO Primate Center, Rijswijk, The Netherlands, employing a "harem type" breeding system. Average weight was 7.5 kg, age varied from nine to 24 years and male to female ratio was two to one. The animals were selected on normal kidney and liver functions and non-injured, relatively large hands. If possible left or right handedness was assessed.

Animal care complied with the "Principles of Laboratory Animal Care" and "the Guide for the care and use of laboratory animals" (NIH-publication no. 80-23, revised 1978).

B.2.2.2 Pre-, per- and postoperative care

Under routine general anaesthesia a continuous axillary plexus block was applied (mepivacaine and adrenaline, 20ml 1% and 0,1 mg respectively per eight hours). Dextran 40 (Rheomacrodex 1,5 gr/kg) was administered as an intravenous drip during and direct after operation for hemodilution. Prophylactic antibiotics were injected intramuscularly during five days. Acetylsalicylic acid 100 mg was also injected on the day of operation.

Following shaving of the arm, the rhesus monkey was placed in a lateral position with the arm abducted. Under sterile conditions and tourniquet control the radial forearm flap was dissected[11, 12] (figure 1). All the important transsected structures were marked during the operation. The flexor, extensor and abductor pollicis longus muscles were cut at the wirst, the adductor was freed from its origin. The following nerves were dissected and cut: the palmar cutaneous branch of the median nerve proximal to the wrist, the median nerve just before the junction of the motor and sensory branches, the motor branch of the ulnar nerve beneath the adductor muscle and the superficial radial nerve branch to the thumb just distal to the site where it pierces the brachioradialis tendon. The branches of the median and superficial radial nerve to the other fingers were left intact. The lateral antebrachial cutaneous nerve was transected proximal to the flap.

Disarticulation was performed at the first carpometacarpal joint. The thumb and radial forearm flap were then developed from distal to proximal, after which the tourniquet was released and careful hemostasis ensured.

Following clamping and transection of the proximal vascular pedicle the amputated part was replanted again by starting with an arthrodesis of the first carpometacarpal joint with a transosseus cerclage wire. The adductor pollicis muscle was reinserted and the tendons of the flexor pollicis longus, the extensor pollicis longus and abductor pollicis longus muscle were reconnected.

Subsequently the nerve ends of the motor branch of the ulnar nerve, the common trunk of the median nerve, the palmar cutaneous nerve and the superficial radial nerve were anastomosed with 10x0 nylon sutures (BV 6, R/Ethilon) under the microscope. The radial digital nerve of the second digit was not restored. As well, the radial artery and the cephalic vein were microsurgically
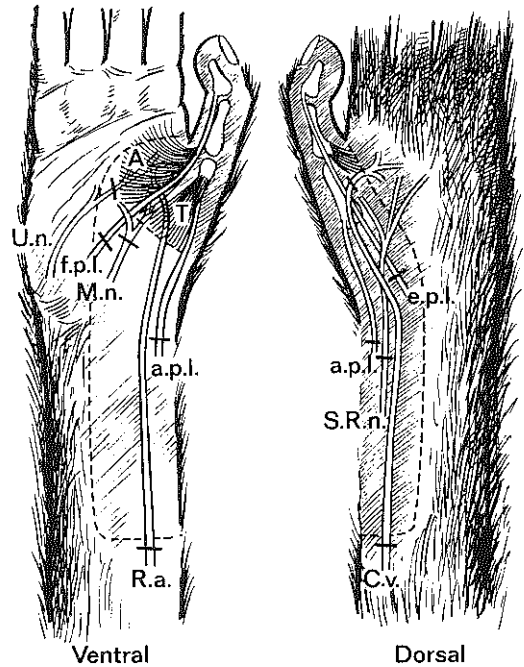

Figure 1. The replant model. The dotted line marks the radial side of the hand with radial forearm flap. Nerves: U.n. = ulnar nerve; M.n. = median nerve; S.R.n. = superfical radial nerve. Muscles: f.p.l. = m. flexor pollicis longus; a.p.l. = m. abductor pollicis longus; e.p.l. = m. extensor pollicis longus. A. = m. adductor pollicis; T. = thenar muscles. Vessels: R.a. = radial artery; C.v. = cephalic vein.

anastomozed end-to-end, with the same suture. The skin was loosely approximated, small drains were placed and the clamps were released (figure 2a + b). The microcirculation of the skin of the replant was monitored after release of the clamps. This was performed with a pin prick test for bleeding as well as continuous Laser Doppler Flowmetry (R/Perimed, PF2, Sweden) recorded, on a marked spot on the forearm flap and the pulp of the thumb.

The monkeys were kept sedated, for general and microcirculatory monitoring.
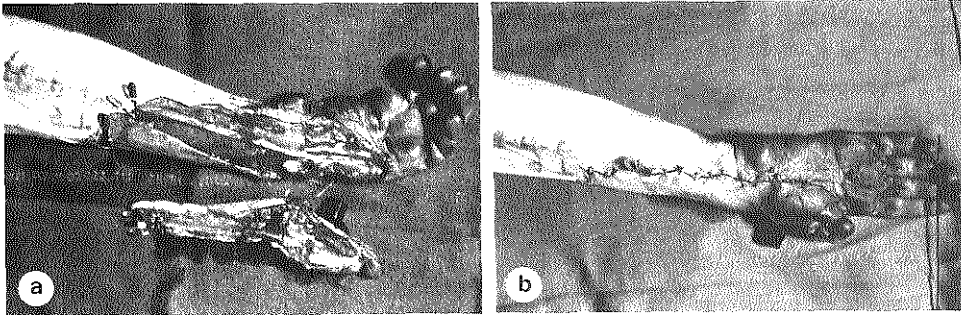
Figure 2a,2b. The radial side of the hand with radial forearm flap following complete transection (a) and after replantation (b).

Consequently a few hours later the arm was dressed and a custom made thermoplast splint was applied, as described previously[13].

Wound inspection was performed two to three times a week under light sedation. Also laser doppler flowmetry was assessed by using interval measurements during two minutes on the same spot as well as contralaterally. Eight weeks postoperatively microcirculatory monitoring was stopped. Dressings and the thermoplast splints were removed as soon as sensory reinnervation could be detected.

B.2.3 Sensory reinnervation

In the initial period a withdrawal reaction to a pin-prick, in the awakening monkey was used as a guide to remove the thermoplast splint and dressings.

Starting one year postoperatively, sensory reinnervation of the replant was assessed monthly over a year, by inducing a withdrawal reflex with a small electric current ("sensiometer"). The contralateral side was used as a control. The conscious monkey was fixed in a specially designed chair (R/Restraint Chair, Primate Products, CA, USA). The "sensiometer" consisted of a stimulation electrode connected to a bipolar 50Hz (sinusoidal) current source stimulator, delivering currents between 0.14 and 1.8 milliAmpère. The electrode had two metal bars, each 1 mm in diameter and with a separation of 1.5 mm.

Stimulation sites on the skin were separated 0.5 cm along three distinct lines covering the replant. The lines were designed in accordance with the expected skin innervation areas of the median, superficial radial, palmar cutaneous branch of the median nerve and the lateral antebrachial cutaneous nerve. The latter was not reconnected and served therefore as a negative control. A current of 1.8 milliAmpère (approximately 1.5 times the threshold for a withdrawal reflex in the contralateral hand) was delivered at each site. False positive reactions were minimized by changing the patterns of stimulation of the skin.

The number of sites demonstrating a withdrawal reaction on stimulation was related to the total number of measuring sites (range 30-40, depending on the size of the replant). Sensory reinnervation was thus expressed as the ratio between these two numbers.

In one monkey (TP), the median nerve was surgically exposed three years postoperatively. Electrical stimulation with a ringelectrode at the tip of the thumb was performed to detect sensory potentials proximal to the anastomosis of the median nerve.

B.2.4 Motor reinnervation

The methods used to examine motor function recovery were electromyography (R/Medelec, PN6), histology and function tasks.

B.2.4.1 Electromyography

The median nerve was stimulated supramaximally and transcutaneously at elbow and wrist and proximal to the anastomosis, under ketamin (R/Fetelar) anaesthesia. The contralateral side served as a control. Surface electrodes were placed in a belly tendon position on the abductor pollicis brevis muscle. To rule out stimulation of the ulnar nerve at the same time a sensory potential was determined at the fifth finger. Latency (in a temperature controlled environment) and amplitude of Compound Motor Action Potentials (CMAP) were compared monthly, starting one year postoperatively, over six months. Latency of CMAP was measured from the moment of stimulation to the peak of the negative deflection in milliseconds. Amplitude of CMAP was measured from the peak of the negative deflection to the baseline, in millivolts. Measurements were related to the contralateral side and expressed as a percentage. To detect latency and amplitude of the CMAP of the thenar muscles by surface electrode, direct stimulation proximal to the anastomosis of the exposed median nerve was performed.

B.2.4.2 Histology

In one monkey (BM) the opponens pollicis and abductor pollicis brevis muscles were removed, 18 months following operation, from origin to insertion from the operated as well as the contralateral hand and snapfrozen in liquid nitrogen chilled isopenthane. The distribution and occurrence of nerve bundles and functional motor end plates over the muscle were inspected microscopically. The activity of acetylcholine- esterase, as described by Karnovsky et al. in 1964[14], was used to detect vital motor end plates. As well, an immunohistochemical staining method specific for neurofilaments[15] was performed on formalin fixed paraffine embedded tissue. This latter method detects the presence of axons within a histologically visible nerve.

B.2.4.3 Function tasks

Functional tasks such as picking up small particles of food with thumb and index finger after bandaging the contralateral hand, were recorded on video.

B.3   Results

B.3.1 Anatomical dissection

Only anatomical findings relevant to the research model are mentioned.

B.3.1.1 Vessels

The distal radial artery divided into a superficial volar branch to form the superficial palmar arch and a dorsal branch which became the first dorsal metacarpal artery. In all dissections, except one, the superficial volar branch ran beneath the flexor pollicis brevis muscle to give off a branch to the thumb. The two dorsal arteries to the thumb were provided by the first dorsal metacarpal artery. A larger venous branch on the dorsum halfway along the first metacarpal provided the main drainage to the cephalic vein (six out of eight).

B.3.1.2 Nerves

The superficial radial nerve varied in four out of eight monkeys. There were either one or two branches to the thumb. The anatomy of the superficial radial nerve was consistent where it pierced the brachioradialis tendon. The median nerve was very consistent at wrist level. Under the distal retinaculum flexorum the median nerve divided mostly into four and sometimes into three branches (two monkeys). The palmar cutaneous branch of the median nerve originated halfway along the forearm and was positioned equally to either radial or ulnar sides of the median nerve. When running ulnarly, this branch crossed the median nerve before reaching the skin of the thenar eminence. The anatomy of the motor branch of the ulnar nerve was very consistent regarding the adductor pollicis muscle (the branch was not dissected further).

Table 1. Results of autologous replantation of the radial side of the hand in rhesus monkeys.

	Results			Complications		
rhesus monkey	wound healing	LDF[2]	sensory recovery	splint decubitus	haemorraghe	death
BM	normal	normal	+	+[3]	-	-
DV	normal	normal	+	-	+[3]	-
TP	normal	normal	+	-	-	-
2585[1]					+	

[1]This monkey did not recover from the anaesthesia and died on day 1 postoperatively due to cardiac arrythmias.
[2]LDF, Laser Doppler Flowmeter to monitor microcirculatory flow of the skin.
[3]Transient.

B.3.1.3 Muscles

The flexor pollicis longus muscle originated from the third tendon of the flexor digitorum profundus just distal to the radiocarpal joint. This tendon is thinner than the tendons to the fingers (in all cases). The extensor pollicis brevis muscle was not encountered in the dissections. The abductor pollicis longus muscle inserted partly onto the radial side of the base of the first metacarpal bone and partly upon the adjacent radial sesamoid. Apart from the adductor pollicis muscle all intrinsic muscles to the thumb originated from the retinaculum flexorum. Co-origins from the carpal bones were present in all thenar muscles.

B.3.2 Replantations

The four replantations of the radial side of the hand were technically successful. Unfortunately, one rhesus monkey (2585) did not recover from the anaesthesia. The monkey died on the first postoperative day due to cardiac arrythmias.   Before actual death, the replant was still well vascularized. Wound healing was uneventful in the other three monkeys, except for a minor and transient postoperative hemorrhage in one monkey and splint decubitus in another (table 1).

Laser doppler flowmetry of the flap and pulp of the thumb showed a higher value in the initial eight days postoperative (approximately twice the contralateral thumb). After the initial period values stabilized and were comparable to the contralateral side.

B.3.3 Sensory reinnervation

Following a reproducible positive withdrawal reflex, from the pin-prick test, the splint was removed in the monkeys BM, DV and TP at the postoperative day 57, 59 and 45, respectively. Complications, such as neurotrophic ulcers or self inflicted damage of the replant, were not seen after removal of the splint.

Figure 3 shows the percentage of sensory reinnervation in the reconnected and control skin areas. In three monkeys, 22 months postoperatively, the percentage of positive withdrawal reactions of the median and superficial radial nerve skin area was 96% with the sensiometer. The area of skin innervated by the palmar cutaneous branch of the median nerve was enclosed in the median nerve area.
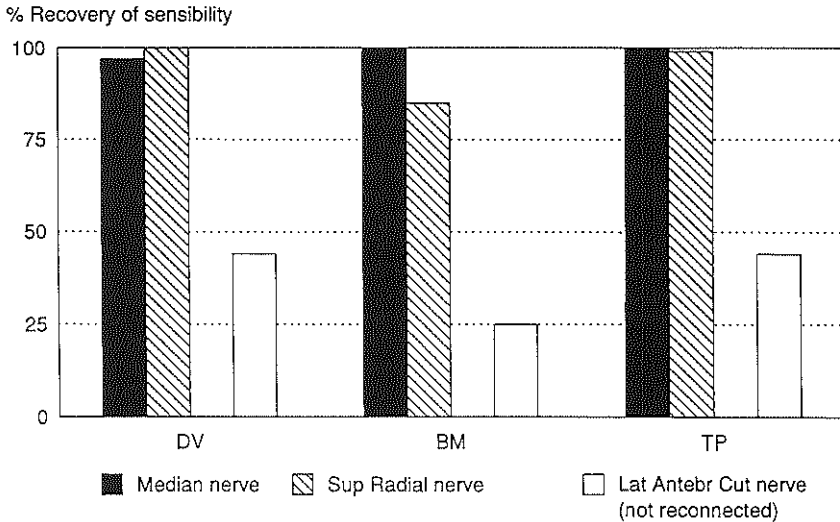
% Recovery of sensibility

Median nerve · Sup Radial nerve · Lat Antebr Cut nerve (not reconnected)

Figure 3. Sensory reinnervation of the radial side of the hand following replantation. In the three monkeys (DV, BM and TP) the median nerve and superficial radial nerve are reconnected. The lateral antebrachial cutaneous nerve is not reconnected.

The area of skin, corresponding to the unreconnected lateral antebrachial cutaneous nerve showed a positive withdrawal reaction percentage of 38% with the same method.

In monkey DV a small area of the tip of the thumb did not react to stimulation.
Sensory potentials of the median nerve in monkey TP were detected and are demonstrated in figure 4a.

B.3.4   Motor reinnervation
B.3.4.1 Electromyography

Mean latency and amplitude of CMAP's of the three replanted thenar muscles following stimulation at the elbow were 93% and 88% respectively, when compared to the latency and amplitudes of CMAP's of the thenar muscles of the contralateral side. Latency and amplitude of CMAP's measured by stimulation of the exposed median nerve of the rhesus monkey (TP) are displayed in figure 4b.
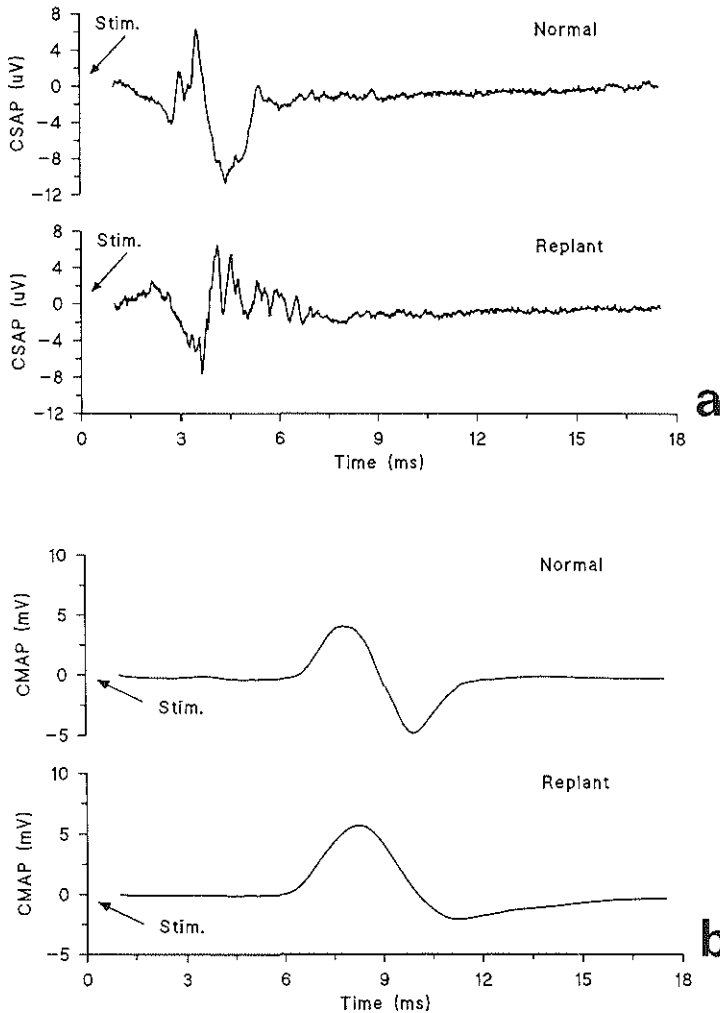
Figure 4a. Compound Sensory Action Potential (CSAP) recorded from the exposed median nerve in the forearm evoked by stimulation of the tip of the thumb by ringelectrode.

The CSAP's of the median nerve of the normal contralateral radial side of the hand and the replanted side in the same monkey are comparable with respect to amplitude and onset of the CSAP. Polyphasia in the CSAP of the median nerve of the replanted side reveals the presence of fibres with delayed conduction. **Figure 4b.** Compound Motor Action Potential (CMAP) of the thenar muscles recorded by surface electrode following stimulation of the exposed median nerve in the forearm. The CMAP's of the thenar muscles of the normal contralateral radial side of the hand and the replanted side are comparable with respect to amplitude and onset of CMAP. The width of the CMAP in the replant indicates the presence of the delayed conduction of a number of fibres.

B.3.4.2 Histology

Immunohistochemical staining for neurofilaments showed the same amount of staining in the replanted muscles as well as on the contralateral side. Staining for acetylcholine-esterase activity as evidence of vital motor end plates revealed a definite amount of staining in the replanted muscles (+) although less than in the contralateral side (+ + +).

B.3.4.2 Function tasks

The monkeys BM and TP could easily pick up small particles of food between thumb and index finger, while monkey DV could not easily do this.

B.4 Discussion

The anatomy and functional use of the hand of the rhesus monkey (Macacca Mulatta) are closely related to the human being[16]. Our anatomical dissections showed that with regard to the vessels, the princeps pollicis artery is mostly small or absent in the rhesus monkey and the superficial palmar arch is dominant. This is in contrast to the human being where the dominant artery to the thumb is mostly the princeps pollicis artery from the deep palmar arch[17, 18].

As emphasis was put on reproducibility of functional recovery of the replant, variations of anatomy regarding nerves had to be minimized. Therefore anastomoses of the nerves were always made at the least variable site. For the median nerve this was just proximal to the junction of sensory and motor branches under the retinaculum flexorum; for the superficial radial nerve just dorsal to the brachioradialis tendon and for the motor branch of the ulnar nerve where it entered the adductor pollicis muscle. Our anatomical dissections of the ulnar nerve did not go beyond the adductor pollicis muscle. When evaluating thenar muscle function in the rhesus monkey however, it is important to know that the motor branch of the ulnar nerve innervates the deep head of the flexor pollicis brevis muscle in 15% and the opponens pollicis muscle in 28%[19]. In the human hand these figures are quite different, 96% and 4%, respectively[20].

In all dissections in the monkey the flexor pollicis longus muscle originated from the deep flexor tendon to the third digit, which is concordant with other primate studies[21]. The muscle tends to separate, however not as completely as in the human being. Clearly the more

independent the flexor pollicis muscle is, the more refined thumb movement will be. The "absence" (or confluence with the abductor longus muscle) of the extensor pollicis brevis muscle and the more proximal insertion of the abductor pollicis longus muscle, observed in our dissections, lead to less active abduction and less stabilization of the metacarpophalangeal joint in relation to the human thumb[22, 23].

The replantations of the radial side of the hand in the rhesus monkey were technically successful and therefore the research model is technically feasible. The advantage of the accessory radial forearm flap was not only to give sufficient skin area for monitoring, but it also enabled more proximal anastomosis of the vessels resulting in a larger diameter (range 0.8 - 1.1 mm) for microvascular surgery. In our replantations arthrodesis of the first carpometacarpal joint was performed, which impairs opposability. As function recovery was an objective, in the allogeneic transplantation project the carpometacarpal joint was left intact and an osteotomy was carried out at the proximal metacarpal level.

In an attempt to predict vascular compromise per- and postoperatively Laser doppler flowmetry was used, as has been well described in microvascular surgery[24, 25]. The inital high flow values after operation are in accordance with clinical replantations (submitted).

This research model resulted in good sensory and motor function recovery as evidenced by the "sensiometer", electromyography, histology and functional tasks. Longitudinal follow-up, assessed in conscious monkeys, demonstrated a mean sensory reinnervation in 96% of the skin area, corresponding to the median and superficial radial nerve. The 38% positive withdrawal reflex in the skin area of the unreconnected lateral antebrachial cutaneous nerve, could possibly be due to collateral nerve sprouting from neighbouring skin regions[26]. Also overlap from median and superficial radial nerve skin distribution areas can cause this positive reaction.

Electromyography, in the second and third year after operation, demonstrated thenar muscle reinnervation. The slightly lower mean amplitude of the CMAP of the thenar muscles and the even less prolonged mean latency of CMAP when compared to the contralateral side (80% and 93%, respectively) are consistent with other studies concerning electromyographical evaluation of muscle reinnervation following a nerve lesion[27, 28, 29].

Further evidence of thenar muscle reinnervation has been provided by histological staining for vital motor end plates[14], although the number of vital motor end plates is less then in the contralateral side. This is similar to recovery after repair in human peripheral nerve injuries[30].

Properly executed functional tasks following replantation of the aforementioned research model was the main objective of this study. In this respect the monkeys BM and TP could easily pick up small particles of food between thumb and index finger, although the radial

digital nerve to the index finger was never restored. In monkey DV, who showed no withdrawal reflex in the tip of the thumb, small particles of food slipped frequently out of the thumb and index finger. This could well be caused by a decrease of sensation of the index finger as well as the tip of the thumb. These observations were recorded by video.

The thumb was obviously reintegrated into the functioning of the hand again after replantation, although the extent of the thenar muscle involvement in relation to the extrinsic muscles could not be quantified or qualified. Dynamic electromyographical studies during function tasks could possibly clarify this problem[31].

In conclusion, despite the differences in the anatomy of the hand between rhesus monkey and man, extrapolation of results seems to be justified. This study has demonstrated the technical feasibility of replantation of the radial side of the hand in the rhesus monkey and has clearly shown evidence of sensory and motor function recovery. The way to allogeneic composite tissue transplantation using the radial side of the hand of the rhesus monkey seems to be opened.

B.4 References

1. TILNEY NL, MILFORD EL, CARPENTER CB, LAZARUS JM, STROM TB, KIRKMAN RL Long-term Results of Cyclosporine Treatment in Renal Transplantation. Transpl Proc, Vol. XVIII, no 2, Suppl 1 (April), pp. 179 - 185, 1986.
2. REICHENSPURNER H, ODELL JA, COOPER DKC, et al: Twenty Years of Heart Transplantation at Groote Schuur Hospital. J Heart Transplantation no 6, pp 17 - 23, 1987.
3. GORDON RD, SHAW BW, IWATSU KIS, et al. Sandimmun' (SIM) in liver transplantation: indications for transplantation and survival. Surg Clin N Amer 66, pp 541 - 556, 1986.
4. FURNAS D.W., BLACK K.S., HEWITT C.W., FRASER L.A. and ACHAUER B.M. Cyclosporine and Long-term Survival of Composite Tissue Allografts (Limb Transplants) in Rats. (With historical notes on the role of Plastic Surgeons in Allotransplantation.) Transpl. Proc., vol. XV, no. 4, pp. 3063 - 3068, 1983.
5. FRITZ W.D., SWARTZ W.M., ROSE S., FUTRELL J.W., KLEIN E. Limb allografts in rats immunosuppressed with Cyclosporin A. Ann. of Surgery, vol. 199, no. 2, pp. 211 - 215, 1984.
6. KIM S.K., AZIZ S., OYER Ph. and HENTZ V.R. Use of Cyclosporine A in Allotransplantation of Rat Limbs. Ann. of Plastic Surg., vol. 12, no. 3, pp. 249-255, 1984.
7. HOVIUS S.E.R., ADRICHEM L.N.A. VAN, HEIJDEN P.M.A. VAN DER, VUZEVSKI V.D., STRIK R. VAN, MEULEN J.C. VAN DER; Postoperative Monitoring of Allogeneic Limb Transplantation in Rats. Annals of Plastic Surgery, vol. 21, no. 6, pp. 559-565, 1988.
8. BUNCKE H.J. and SCHULZ W.P. Experimental digital amputation and reimplantation. Plast. & Reconstr. Surg., vol. 36, no. 1, pp. 62 - 70, 1965.
9. DANIEL R.K., EGERSZEGI E.P., SAMULACK D.D., SKANES S.E., DYKES R.W., RENNIE W.R.J. Tissue transplants in primates for upper extremity reconstruction: A preliminary report. Journ. of Hand Surgery, Am. vol. 11a, no. 1, pp. 1 - 8, 1986.
10. STARK G.B., SWARTZ W.M., NARAYANAN K., MOLLER A.R. Hand Transplantation in

Baboons. Transpl. Proc., vol. 19, no. 5, pp. 3968 - 3971, 1987.

11. WEBSTER MHC, SOUTAR DS; Practical Guide to Free Tissue Transfer. Butterworths, London, 1986.

12. HOVIUS S.E.R., SLUIMERS J.E., ADRICHEM L.N.A. VAN, VAANDRAGER J.M., WIJTHOFF S.J.M., MEULEN J.C. VAN DER; The radial forearm flap. The Neth. J. of Surgery, 40-3, pp. 69 - 75, 1988.

13. HOVIUS SER, STEVENS HPJD, PETERS B and NIEROP PWM VAN; A protective thermoplast splint for the forearm and hand in non-human primates. Lab. Animals, no. 25, pp. 1 - 3, 1991.

14. KARNOVSKY M.I., ROOTS L. A "direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem., no. 12, pp. 219 - 221, 1964.

15. KLüCK P., MUYEN G.N.P. VAN, KAMP A.W.M. VAN DER, TIBBOEL D., HOORN W.A. VAN, WARNAAR S.O., MOLENAAR J.C. Hirschsprungs disease studied with monoclonal anti-neurofilament antibodies on tissue sections. Lancet, pp. 652 - 654, 1984.

16. HARTMAN C.G. and STRAUS W.L. Eds. The Anatomy of the Rhesus Monkey. Hafner Publishing Co., New York, 1933.

17. IKEDA A., UGAWA A., KAZIHARA Y. et al; Arterial patterns in the hand based on a three-dimensional analysis of 220 cadaver hands. J Hand Surg, vol. 13A, no. 4, pp. 501 - 509, 1988.

18. ROJECKI F. Sur la Circulation Arterielle chez le Macacus Cynomolgus et le Macacus Sinicus. J. Anat. Paris, no. 25, pp. 343 - 386, 1889.

19. AKIYAMI K. The nerves of the Hand of the Macacus Cyclopsis. Okajimas Folia Anatomica Japonica, no. 34, pp. 641 - 655, 1960.

20. BONNEL F. and VILA R.M. Anatomical Study of the Ulnar Nerve in the Hand. The Journ. of Hand Surg., vol. 10B, no. 2, pp. 165 - 168, 1985

21. LANDSMEER J.M.F. A comparison of fingers and hand in Varanus, Opossum and Primates. In: Acta Morphologica Neerlando-Scandinavica, vol. 24, no. 3, pp. 193 - 221, 1986.

22. NAPIER J.R. Prehensility and opposability in the hands of primates. Symp. Zool. Soc. Lond., no. 5, pp. 115 - 133, 1961.

23. BURTON F.D. An analysis of the muscular limitation on opposability in seven species of Cercopithecinae. Am. J. Phys. Anthrop., no. 36, pp. 169 - 188, 1972.

24. HEDEN P.G., HAMILTON R., ARNANDER C., JURELL G. Laser Doppler Surveillance of the Circulation of Free Flaps and Replanted Digits. Microsurgery, no. 6, pp. 11 - 19, 1985.

25. JENKINS S., SEPKA R., BARWICK W.J. Routine Use of Laser Doppler Flowmetry for Monitoring Autologous Tissue Transplants. Annals of Plast. Surg., vol. 21, no. 5, pp. 423 - 426, 1988.

26. HORCH K.W. Absence of functional collateral sprouting of mechanoreceptor axons into denervated areas of mammalian skin. Experimental Neurology, no. 74, pp. 313 - 317, 1981.

27. BALLENTYNE JP, CAMPBELL MJ. Electrophysiological study after surgical repair of sectioned human peripheral nerves. J of Neurology, Neurosurg and Psychiatry, no. 36, pp. 797 - 805, 1973.

28. DAVIS LA, GORDON T, HOFFNER JA, et al; Compound action potentials recorded from mammalian peripheral nerves following ligation or resuturing. J Physiol: 285: pp. 543 - 559, 1978.

29. BUCHTAL F., KÜHL V. Nerve conduction, tactile sensibility, and the electromyogram after suture or compression of peripheral nerve: a longitudinal study in man. Journal of Neurology, Neurosurgery, and Psychiatry, no. 42, pp. 436 - 451, 1979.

30. LUNDBORG G. Nerve injury and nerve repair. Churchill Livingstone. Edinburgh, London, Melbourne and New York, 1988.

31. COONEY W.P., AN K.A., DAUBE J.R. and ASKEW L.J. Electromyographic analysis of the thumb: a study of isometric forces in pinch and grasp. The Journal of Hand Surg. vol. 10A, pp. 202 - 210, 1985.

CHAPTER II: INTRODUCTORY STUDIES
STUDY C : A protective thermoplast splint for the forearm and hand in primates

S.E.R. Hovius & H.P.J.D. Stevens

C.1 Introduction

Over the centuries materials used to immobilise extremities have developed from parallel fixed pieces of wood to circular plaster of Paris and thermoplastic splints. The last of these materials has gained wide acceptance due to its light weight, strength, durability, easy moulding and remoulding in different positions. Even under extreme circumstances such as immobilisation of extremities in nonhuman primates, thermoplastic splints have proved to be adequate[1]. In this study perforated thermoplast was used to protect the forearm and hand of rhesus monkeys after experimental surgery.

C.2   Materials and Methods

C.2.1 Animals and housing

Rhesus monkeys (Macaca mulatta) were born and raised in the TNO Primate Center, Rijswijk employing a harem type breeding system. The animals were individually housed in metal cages of 0.6 x 0.6 x 0.8 m³. Average weight was 7.5 kg. Age varied from 9 to 24 years and male to female ratio was 2:1.

C.2.2 Description of the splint

The arm of the anaesthetised monkey was shaved to prevent the damage to the skin which often occurs when hairy skin is covered by a dressing. After wrapping the individual fingers, hand and arm up to the axilla with synthetic wadding (Soffban', T.J. Smith and Nephew Ltd, Hoofddorp, The Netherlands) secured with elastic bandage (Bandafix', International Medical products, Zutphen, The Netherlands), a paper template was made to assess the size and form of the thermoplastic sheet. The thermoplastic sheet (San-Splint' XR, T.J. Smith and Nephew Ltd, Hoofddorp, The Netherlands) selected had preperforations, to allow some ventilation. After cutting the sheet to an appropriate size it was heated in a water bath to approximately 70 °C. The surface cools down rapidly to approximately 40 °C whilst retaining its capacity to be moulded.

Figure 1. A rhesus monkey, wearing the thermoplastic splint.

The cooled but malleable sheet was laid over the radial side of the outstretched arm of the monkey after the most proximal end of the sheet had been folded double to avoid a sharp edge and for strength. During hardening of the material the arm was flexed at the elbow to 110° and the splint was moulded to its final form by joining the edges at the ulnar side. Finally the one piece splint was fixed either with 5 nuts and bolts at the broad ulnar margin or with circular tape (Fig. 1). Duration of the entire procedure described was approximately 45 minutes.

C.3 Results

Thirty nine splints were evaluated, each custom made for a different monkey. Duration of splinting varied from 12 to 79 days. Complications consisted of transient pressure ulcers in 3 rhesus monkeys, spontaneous removal of splint in 2 cases and twisting of the arm within the splint in one monkey. In none of the cases did breakdown of the splint occur. However in 2 cases the proximal side of the splint had to be stengthened after partial damage due to biting.

C.4 Discussion

A wide variety of materials is used for splinting in humans[2-4]. However due to the nature of caged nonhuman primates the use of most of these materials is inadvisable. For example a

plaster of Paris splint would be too heavy and too susceptible to breakdown by wetting or biting. Also in the case of pressure ulcers local adjustment of the splint would not be possible necessitating a total replacement. A splint on a glass fibre base would be too rigid, too sharp, too expensive and too laborious to make. Furthermore it would make removal of the cast for inspection of the arm very difficult. The thermoplastic splint, however, could be easily moulded and reshaped if necessary. It was light in weight with an average of 300 grams and proved to be strong and durable. Even under difficult circumstances following microvascular surgery or in an immunosuppressed animal with a wound highly susceptible to infection, the operation site and wound could be adequately protected.

Pressure ulcers ocurred in three cases at the elbow and ulnar side of the wrist. Local adjustment of the splint could easily be performed with the heatgun and resulted in the healing of all ulcers. Fixation with either nuts and bolts or circular tape appeared to be adequate in most of the cases, apart from two spontaneous removals. These were probably due to a combination of lack of flexion at the elbow and too wide an opening at the upper arm. In one case the arm was twisted within the splint, presumably as a result of unusual movements during restraint of the monkey and a lack of flexion at the elbow. A slight increase in the degree of flexion of the splint proved to be adequate to prevent recurrence.

Preconditioning of the monkey to the splint was not necessary. Removal and refixation of the one piece splint could easiliy be performed by one person. It could be done frequently without damaging the splint, even if the splint had not been used for half a year or more.

Although thermoplast has not been compared with other materials it seems an appropriate material for provison of immobilisation and protection of the arm and hand in the non-human primate.

C.5 References

1. Rose BW, Mackinnon SE, Dellon AL, Snyder RA: Design of a protective splint for nonhuman primate extremities. Lab Animal Sc 1983; 33(3): 306.
2. Malick M: Manual on dynamic hand splinting with thermoplastic materials. Pittsburgh: Harmaville Rehabilitation Center. 1978.
3. Freuler F, Wiedmer U, Bianchini D: Cast manual for adult and children. Berlin; New York; Springler Verlag 1979.
4. Fess EE, Philips CA: Hand Splinting: Principles and Methods (2nd edition). St. Louis: C.V. Mosby Co, 1987.

CHAPTER II: INTRODUCTORY STUDIES

STUDY D   : **In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive cells**

H.P.J.D. Stevens

D.1 Introduction

Selective intervention by monoclonal antibodies (MAbs) directed against T cells (CD3+ lymphocytes) has demonstrated to be effective in suppressing cellular immune responses, both in man[1] and rhesus monkey[2]. Also MAbs specific for CD4 or CD8 positive cells which are both involved in the process of graft rejection[3], can prolong skin allograft survival in rhesus monkeys[4,5]. Furthermore, MHC antigens were shown play a key role in cellular immune responses like allotransplant rejection[6].

To investigate the importance of these surface markers in the process of graft rejection MAbs specific for these antigens have, so far, mainly been tested individually. This study, however, focussed on the immuno-suppressive effect of a combination of MAbs specific for CD3+, CD4+, CD8+ and MHC class II positive cells in a rhesus monkey skin allograft model to investigate whether a potent immunosuppressive cocktail of monoclonal antibodies could be established.

D.2   Materials and methods

D.2.1 Experimental design

Skin grafts were exchanged between non-immunized unrelated outbred rhesus monkeys (Macaca mulatta) that were born and raised in the TNO Primate Center, Rijswijk. Donor and recipients were mismatched for MHC class I and II antigens. The number of class I mismatches in groups 1 to 5 was one, while in groups 6 to 8 it was four.

The skin graft experiment concerned eight different experimental groups (Table 1).

D.2.2 Monoclonal antibodies used for in vivo treatment

The following MAbs were used for in vivo treatment: FN18[7], IgG1 and 5B11, IgG2a both specific for CD3; RIV6 and RIV7, both IgG2a and specific for CD4[8], FK18, IgG3, specific for CD8[8,9]; B8.12[10], IgG2b and 7.5.10.1[11], IgG2a both are specific for HLA-DR. The dosages used in this study are given in table 1. Recipients received their MAbs as an i.v. bolus injection for the duration of 10 days starting day -1, one day before skin transplantation. All MAbs used in this study were crossreactive with rhesus lymphocytes. They were purified by absorption to protein A and sterilized, prior to in vivo administration.

After the first injection of the cocktail of MAbs or its anti-T cell fraction in the above

mentioned dose or a 20 to 40 % of this dose, minor side effects occurred in two out of eight cases (gall expectoration in one case, short period of drowsiness in another). In one out of eight cases, shock like symptoms developed and the monkey was euthanized.

D.2.3 Immunological monitoring

Levels of circulating injected MAbs and titers of antibody formation against the injected MAbs were determined using an ELISA technique.

All allograft recipients were monitored frequently with respect to total leucocyte and lymphocyte counts and relative numbers of CD3+, CD4+, CD8+ and MHC class II+ cells, using an indirect immunofluorescence technique and analysis by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

Immunohistological staining of punch biopsy specimens taken at regular intervals from the allogeneic skin in group 8, was performed with MAbs specific for MHC class I and II antigen expression, CD3+, CD4+ and CD8+ cells. Parallel hematoxylin and azofloxine-stained sections were processed in a routine matter.

D.3 Results and discussion

Allogeneic skin grafting in rhesus monkeys, treated prophylactically with MAbs against lymphocyte (sub)populations, is a well established animal model to test immunosuppressive potencies of such antibodies in the non-human primate[12]. Previous studies demonstrated that FN18 specific for CD3+, RIV7 specific for CD4+ and FK18 specific for CD8+ cells could prolong allograft survival in a rhesus monkey skin transplantation model (Table 1)[7,8]. Further results concern groups 6, 7 and 8. A combination B8.12 and 7.5.10.1 (group 7) could prolong skin graft survival to 11.8 +/- 0.3 days compared to the control group of 8.3 +/- 0.7 days. However, the combination of seven MAbs specific for CD3+, CD4+, CD8+ and MHC class II positive cells (group 8), prolonged skin graft survival to 19.3 +/- 1.3 days, compared to the same control group (see Table 1 and materials and methods section for description of the MAbs). Results suggest, that the combination these MAbs tends to induce addition of their individual suppressive effects on the immune response. This tendency of addition is clearly present, even though the increase of the number of mismatches has a significant effect on the reduction of the skin allograft survival times (groups 1 and 6, Mann-Whitney U test, p=0.01).

In all cases, the daily injected doses of MAbs were sufficient to maintain significant serum levels until the next injection was given 24 hours later. However, approximately 7 days after operation the appearance of anti-mouse-antibodies neutralized the injected MAbs.

Skin graft recipients treated with the combination of MAbs specific for MHC class II antigens (group 7) showed a slight lymphopenia which lasted for 1 to 2 days. 24 Hours after the first injection, the MHC class II positive cells were coated by the injected MAb. Coating

Table 1. Skin graft survival times in rhesus monkeys treated with MAbs specific for CD3+, CD4+, CD8+ and/or MHC class II positive cells

Groups[a]	Injected dose MAb(s)	(mg/kg)	Target	(n)	Subset effect	Individual survival times (days)	MST +/- SE
1.	-/-		control	42			9.3 +/- 0.3
2.	FN18	1	CD3	3	elimination, modulation	16 16 18	16.7 +/- 0.7
3.	RIV6	1	CD4	2	coating	9 9	9.0
4.	RIV7	1	CD4	4	coating	8.5 9 10 19	11.6 +/- 4.3
5.	FK18	1	CD8	4	elimination	8 12.5 14 15	12.4 +/- 2.7
6.	-/-		control	10			8.3 +/- 0.7
7.	B8.12	1.4	MHCclassII	2	coating	11.5 12	11.8 +/- 0.3
	7.5.10.1	1.4			coating		
8.	5B11	0.5	CD3	2		18 20.5	19.3 +/- 1.3
	FN18	0.5	CD3				
	RIV6	0.5	CD4				
	RIV7	0.5	CD4				
	FK18	0.5	CD8				
	B8.12	1	MHCclassII				
	7.5.10.1	1	MHCclassII				

[a]The number of MHC class I mismatches in groups 1 to 5 was one, while in groups 6 to 8 it was four.
[b]MST +/- SE; mean skin graft survival time +/- standard error

gradually decreased within 6 days after the first injection, while relative numbers of MHC class II positive cells did not change significantly. No fluctuations in CD3+, CD4+ or CD8+ cells could be seen. Skin graft recipients treated with the cocktail of seven MAbs showed a lymphopenia to 11% of pretreatment levels which was sustained till approximately five days after the first injection. Elimination of peripheral blood lymphocytes mainly concerned T cells of the CD3 and/or CD8-positive phenotype. Almost all remaining lymphocytes were coated directly after injection. Coating gradually decreased and lasted until approximately day 5. Modulation could be observed for the CD3 surface antigens. It seems likely that the individual effects that MAbs have on lymphocyte subset distributions, remain present, when combined to a cocktail (Table 1).

Results show that the combination of MAbs specific for CD3+, CD4+, CD8+ and MHC class II positive cells has a strong immunosuppressive effect which is comparable to polyclonal ATG preparations (Balner, Van Vreeswijk et al, unpublished results). Addition of MAbs specific for CD4, CD8, and MHC class II antigens on top of anti-CD3 specific MAbs, seems to have an additive effect on prolongation of allograft survival times. This might be of practical use in the treatment of patients with a rejection crisis of their allotransplant.

D.4 References

1. Cosimi AB, Burton RT, Colvin RB, et al; Transplantation 32:535, 1981
2. Nooij FJM and Jonker M; Eur J Immunol 17:1089, 1987
3. Loveland BE, McKenzie IFC; Transplantation 33(3):217, 1982
4. Jonker M, Malissen B, Vreeswijk W van, etal; Transpl Proc 15(1):635, 1983
5. Jonker M, Nooij FJM and Chatenoud L; Transpl Proc 18(5):1287, 1986
6. Milton AD and Fabre JW; J Exp Med 161:98, 1985
7. Nooij FJM and Jonker M, Eur J Immunol 17:1089, 1987
8. Jonker M, Nooij FJM and Chatenoud L; Transplant Proc 18(5):1287, 1986
9. Koning F, Fifth European Immunology meeting, 1982
10. Jonker M, Slingerland W: In Leucocyte typing IV. Knapp W et al (eds): Oxford University Press, 1990; p1058.
11. Koning F, Schreuder GMT, Bontrop R, etal; Disease Markers 2:75, 1984

CHAPTER II: INTRODUCTORY STUDIES
STUDY E   : Monoclonal antibodies for immunohistochemical labelling of immunocompetent cells in frozen sections of rhesus monkey tissues

H.P.J.D. Stevens

E.1 Introduction

Much of our understanding of the immune response depends upon the study of cell interactions in animal models of human immunopathological conditions. The close phylogenetic relationship between man and rhesus monkey and the high degree of similarity of their immune systems makes this species a useful experimental animal for preclinical research, especially in the fields of bone-marrow and allogeneic organ transplantation [17,9] autoimmune diseases [30,3] and more recently in AIDS research [12,33]. Similarities in function and structure of rhesus major histocompatibility complex (MHC) class I and II antigens [49,41] as well as of many lymphocyte antigens [19,34] and cytokines [46] made it possible to use human specific reagents like monoclonal antibodies (MAbs) for the study of the immune response in this species. However, not all MAbs that had been demonstrated to be crossreactive using techniques like fluorescent activated cell sorting (FACS), immunoprecipitation and bioassays, appeared to be effective in immunohistochemical studies, using an indirect immunoperoxidase technique. As a prerequisite for further studies of nonhuman primate immunohistology, this report describes the labelling patterns of several MAbs specific for MHC class I, II-DR, -DQ and -DP antigens, the leucocyte markers CD1, CD2, CD3, CD4, CD8, CD14, CD16, CD25, CD57; a proliferation associated nuclear antigen (Ki67) and the lymphokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) on frozen sections in a number of rhesus monkey tissues.

E.2 Materials and methods
E.2.1 Tissues for (immuno)histology

Lymph node, thymus, liver and skin biopsies were taken from different rhesus monkeys, all born and raised in the Rijswijk Primate Center. Skin biopsies originated from allogeneic partial hand transplant recipients, treated with cyclosporine A and prednisolone. Biopsies were taken during onset of rejection to ensure activation of cell surface markers and the presence of activated (host) lymphocytes and macrophages [45]. After harvesting, each biopsy was cut in half, longitudinally. One half was fixed in buffered formalin and processed routinely for histology on hematoxylin and azofloxine-stained sections. For parallel immunohistochemical studies the other half was snap-frozen in liquid nitrogen chilled isopentane. Cryostat sections of 6 μm thickness were cut, air-dried, fixed in acetone and stored at -70°C until use.

Table 1. Crossreactivity of anti-human mAbs with their rhesus homologous counterpart

Antibody	Target[#]	Reference	Technique	Reference	Prep.[#]
W6/32[a]	MHC-A,B	4	IPrecip	37	I
HLA-DR[b]	MHC-DR	31	FACS	2	I
B8.12[c]	MHC-DR	36	FACS	36	I
7.5.10.1[d]	MHC-DR,DQ,DP	28	FACS	47	I
Genox 3.53[e]	MHC-DQw1	10	FACS	23	I
anti-Leu10[b]	MHC-DQ	11	IPrecip	8	I
anti-HLA-DP[b]	MHC-DP	50	IPrecip	8	I
anti-Leu6[b]	CD1	52	immPO	22	I
T11[f]	CD2	21	FACS	26	II
anti-Leu5b[b]	CD2	5	FACS	26	I
5B11[g]	CD3	26	FACS	26	I
FN18[h]	CD3	36	FACS	36	I
OKT4+4A[i]	CD4	29	FACS	26	I
M-T310[j]	CD4	39	FACS	26	III
RIV6[k]	CD4	25	FACS	26	I
RIV7[k]	CD4	25	FACS	26	I
GM9[h]	CD8	24	FACS	24	I
FK18[d]	CD8	27	FACS	26	I
JMLH14[l]	CD14	15	FACS	26	III
anti-LeuM3[b]	CD14	13	immPO	2	I
WT14[m]	CD14	6	immPO	hl[#]	II
UCHM1[n]	CD14A	20	FACS	26	III
My4[o]	CD14	18	immPO	hl[#]	I
anti-Leu11b[b]	CD16	38	immPO	hl[#]	I
2A3[b]	CD25	48	FACS	26	I
33B3.1[p]	CD25	44	FACS	26	I
anti-Leu7[b]	CD57	1	immPO	hl[#]	I

Table 1. Continued.

Antibody	Target[#]	Reference	Technique	Reference	Prep.[#]
MD1[d]	IFN-γ	35	bioassay[r]	46	I
61E71[a]	TNF-α	32	bioassay[s]	46	I
Ki67[f]	hu-nucl. Ag	16	immPO	hl[#]	II

Sourse: [a]Seralab, Crawley Down, UK; [b]Becton Dickinson, Sunnyvale, CA, USA; [c]Dr C. Mawas, Marseille, France; [d]Dr F. Koning, Bloodbank, University Hospital, Leiden, The Netherlands; [e]Dr W.F.Dodmer, London, UK; [f]Dakopatts, Glostrup, Denmark; [g]Dr R. van Lier, Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; [h]Dr M. Jonker, Institute for Applied Radiobiology and Immunology, Rijswijk, The Netherlands; [i]Ortho Diagnostic Systems, Raritan, NJ, USA; [j]Dr E.P. Rieber, Munchen, FRG; [k]RIVM, Nijmegen, The Netherlands; [l]Dr M.M. Yokoyama, Fukuoka, Japan; [m]Dr W. Tax, Nijmegen, The Netherlands; [n]Dr P.C.L Beverly, London, England; [o]Coulter Clone, Hialeah, FL, USA; [p]Dr J.P Soulillou, Nantes, France; [q]Dr W.A. Buurman, Biomedical Center, University of Limburg, The Netherlands.
Technique: FACS: indirect immunofluorescence staining technique and analysis by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA); IPrecip: immunoprecipitation; immPO: indirect immunoperoxidase staining; bioassay[r]: cytopathic inhibition assay in Hep-2 cells with vesicular stomatitis virus as challenge; bioassay[s]: bioassay with a murine WEHI 164 clone 13 fibrosarcoma cell line.
Material used in this study: I) ascites purified by protein A affinity chromatography; II) tissue culture supernatant; III) unpurified ascites;
[#]**Abbreviations used: Prep.** = Antibody preparation used for this study; **MHC** = Major Histocompatibility Complex antigens class I or II; **CD** = Cluster of Differentiation, as defined during the Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989); **IFN-γ** = Interferon gamma; **TNF-α** = Tumor Necrosis Factor alpha; **hu-nucl. Ag** = human nuclear antigen; **hl** = hoc loci, this study.

E.2.2 Monoclonal antibodies used for immunohistochemical staining

All MAbs used in this study are listed in table 1. Twenty-five of the tested MAbs were known for their crossreactivity with the homologous rhesus counterpart antigens in indirect immuno-fluorescence studies, immunoprecipitation studies or in bioassays. Five MAbs of which crossreactivity had not been assessed previously, were also included in this study, as their target structures are of particular interest. Table 1 lists the specificity and original source of the antibodies, the technique (and reference) previously used for demonstrating crossreactivity with rhesus monkey cells as well as the method of purification of the

antibody used for this study. Except for the MAbs FN18 [36] and GM9 [24], that were developed against rhesus antigens, all other MAbs used in this study were originally raised against human antigens.

E.2.3 Immunohistochemical staining technique.

Incubation was performed using an indirect immuno-peroxidase staining technique. Briefly, slides were thawed at room temperature for 15 min, washed with PBS, and then incubated with the tested MAb (Table 1) for 60 min at room temperature. Slides were washed again and subsequently incubated for 30 min with horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS containing 5% heat-inactivated (30 min., 56°C) normal rhesus serum and 5% normal rabbit serum. After rinsing, conjugate binding was visualized with a solution of 0.5 mg/ml 3,3,-diaminobenzidine tetrahydrochloride (Fluka Chemika, FRG) and 0.03% H_2O_2 in PBS. Slides were washed and counterstained with Mayer's hematoxylin for 1 minute, dehydrated and finally embedded in Malinol (Chroma Gesellschaft, Kongen, FRG). Of each MAb a titration with a minimum of 4 different dilutions was performed. In control incubations the primary antibody was omitted. Immunohistochemical reactivity of each MAb was scored semiquantitatively: - for absence of, + for low, + + for moderate and + + + for high intensity of staining. Both staining of the relevant target structure and aspecific background staining were assessed. Only the results of the most optimal dilution of each tested MAb are presented in tables 2 through 5.

E.4 Results

In tables 2 through 5, the immunohistochemical reactivity of 30 different MAbs with various rhesus tissues is presented.

In table 2, results are given of seven MAbs specific for MHC class I and II antigens, tested on lymph node and allogeneic skin. W6/32, anti-HLA-DR and B8.12, the former specific for MHC class I antigens and the latter two specific for MHC class II-DR antigens, proved to stain target structures intensely, without significant non-specific background staining. Intense cellular staining of lymphocytes could be seen both in lymph nodes and cellular infiltrates of the skin. Moreover, a clear expression of MHC class I and II-DR antigens by epidermal and dermal cells in a skin biopsy was also observed (Fig. 1a).

Table 2. Immunohistochemical reactivity[a] of mAbs specific for Major Histocompatibility Complex class I and II antigens in the rhesus monkey.

MAb	Target: MHC class	REACTIVITY IN Lymph node	Skin
W6/32	A,B	+ + +/-	+ + +/-
anti-HLA-DR	DR	+ + +/-	+ + +/-
B8.12	DR	+ + +/-	+ + +/-
7.5.10.1	DR,DQ,DP	+/-	+/+
Genox 3.53	DQw1	+ + +/-	+ + +/+
anti-Leu10	DQ	+ +/-	-/-
anti-HLA-DP	DP	+ +/-	-/-

[a]Reactivity of the tested mAb is scored semiquantitatively: - for absence of, + for low, + + for moderate and + + + for high intensity of staining. Both staining of the relevant target structure and aspecific background staining were assessed and are presented in this order, divided by a slash. Only the results of the most favorable dilution of each tested mAb are presented.

MAbs specific for MHC class II-DP and -DQ antigens showed a moderate staining reaction with a minority of lymphoid cells in lymph nodes. These cells were exclusively found diffusely in the paracortical areas of lymph nodes.

In table 3, 12 MAbs are listed, specific for T cell antigens CD2, CD3, CD4, CD8 or CD25. These MAbs were tested on lymph node, thymus and allogeneic skin. T11 most specificly and intensively stained mature T cells, whereas anti-Leu5b did not. Though all MAbs specific for CD3 also stained lymphocytes intensively, slight background staining was noted in all tissue tested. FN18 strongly crossreacted with hair follicles in skin specimens. The combination of OKT4 and OKT4A appeared to be highly reactive with the CD4+ T cell subset in lymph nodes as well as in thymus and skin biopsies. The MAb GM9, directed against rhesus CD8, yielded more specific staining results than FK18, though both MAbs showed intense target staining (Fig. 1b). The MAb 2A3 was found to stain with lymphocytes expressing the CD25 marker in lymph nodes and thymus. Expression of the CD25 surface antigen was, however, not detectable on skin allograft infiltrating lymphocytes and histiocytes with this MAb. With 33B3.1 no target staining was detectable.

Table 3. Immunohistochemical reactivity[a] of mAbs specific for
lymphocytes markers in the rhesus monkey.

| MAb | Target | REACTIVITY IN | | |
		Lymph node	Thymus	Skin
T11	CD2	+ + +/-	+ + +/-	+ + +/-
anti-Leu5b	CD2	-/-	-/-	-/-
5B11	CD3	+ + +/+	+ + +/+	+ + +/+
FN18	CD3	+ + +/+	+ + +/+	+ + +/+ +[b]
OKT4+4A	CD4	+ + +/-	+ + +/-	+ + +/-
M-T310	CD4	+/-	+/-	+/-
RIV6	CD4	-/-	-/-	-/-
RIV7	CD4	-/-	-/-	-/-
GM9	CD8	+ + +/-	+ + +/-	+ + +/-
FK18	CD8	+ + +/+	+ + +/+	+ + +/+
2A3	CD25	+ + +/+	+ +/-	+/+
33B3.1	CD25	-/-	-/-	+/+

[a]See legend Table 2.
[b]In this case background staining also included the staining of hair follicles.

In table 4, eight MAbs are listed, specific for the leucocyte markers CD1, CD14, CD16 and CD57, respectively. These MAbs were tested on lymph node, liver and grafted allogeneic skin. The MAb anti-Leu6 (CD1-specific) very intensively stained dendritic cells that were mainly located in the epidermis. JML-H14 was the only one out of five MAbs specific for CD14(A), that proved to stain effectively Kupffer cells in the liver and histiocytes in lymph nodes and in infiltrating lymphocytes of allogeneic skin grafts (Fig. 1c). Anti-Leu7 (CD57-specific) showed a moderate staining reaction on small numbers of lymphoid cells in lymph nodes. CD16 could not be stained immunohistochemically with anti-Leu11b, not in lymphoid tissue nor in allogeneic skin during rejection.

Table 4. Immunohistochemical reactivity[a] of MAbs specific for leucocyte markers
CD1, CD14, CD14A, CD16 and CD57, in the rhesus monkey.

MAb	Target	REACTIVITY IN		
		Lymph node	Liver	Skin
anti-Leu6	CD1	+ + +/-	nt[b]	+ + +/-
JML-H14	CD14	+ + +/-	+ + +/-	+ + +/-
anti-LeuM3	CD14	+ + +/+	+/-	+/+
WT14	CD14	+/-	+ +/-	-/-
UCHM1	CD14A	-/-	+/-	+ +/-
My4	CD14	-/-	+ +/-	+/+
anti-Leu11b	CD16	-/-	nt	+/+
anti-Leu7	CD57	+ + +/+	nt	+/+

[a]See legend Table 2.
[b]nt = not tested

Table 5. Immunohistochemical reactivity[a] of MAbs specific for lymphokines
and proliferating cells in the rhesus monkey.

MAb	Target	REACTIVITY IN	
		Lymph node	Skin
MD1	IFN-γ	+ + +/+ +	+/+
61E71	TNF-α	+ + +/+	+/+
Ki67[b]	hu-nucl. Ag[c]	+ + +/-	+ + +/+

[a]See legend Table 2.
[b]In thymus, positive cells were mainly located in the cortex and rarely in the medulla.
[c]hu-nucl. Ag: human nuclear antigen

In table 5, results are given with regard to MAbs specific for IFN-γ, TNF-α and human nuclear antigen, tested on lymph node and skin. Several cells showed intense pericellular staining with MD1 and 61E71, respectively specific for IFN-γ and TNF-α. However, the incidence of reactive cells during skin allograft rejection was very low.
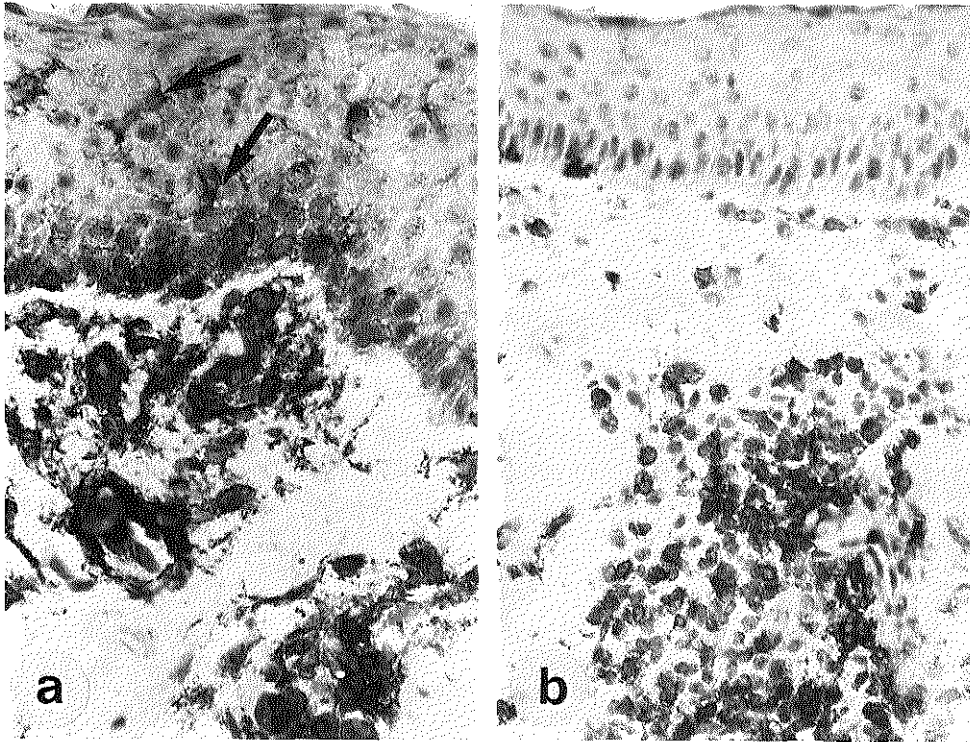
Figure 1a. Staining with anti-HLA-DR of an allogeneic skin biopsy during rejection demonstrated the extensive MHC class II-DR antigens expression on epidermal and dermal cells and lymphocytes in the rejection infiltrate. Also Langerhans cells were stained (arrow).
Figure 1b. Staining with the MAb GM9 directed against rhesus CD8 lymphocytes yielded intense pericellular target staining in an allogeneic skin biopsy during rejection.

Also an aspecific background staining and diffuse intercellular staining was observed in skin sections. Ki67, directed against a proliferation associated nuclear antigen in human tissues, yielded a clear nuclear staining reaction of cells in thymus, lymph nodes and skin (Fig. 1d). In thymus, positive cells were mainly located in the cortex and rarely in the medulla. In peripheral lymphoid tissue, many cells in germinal centers were reactive. In skin biopsies, Ki67 often reacted with the cytoplasm of the basal epithelial cells of epidermis and hair follicles, but preferentially labelled nuclei of the basal cell layer.
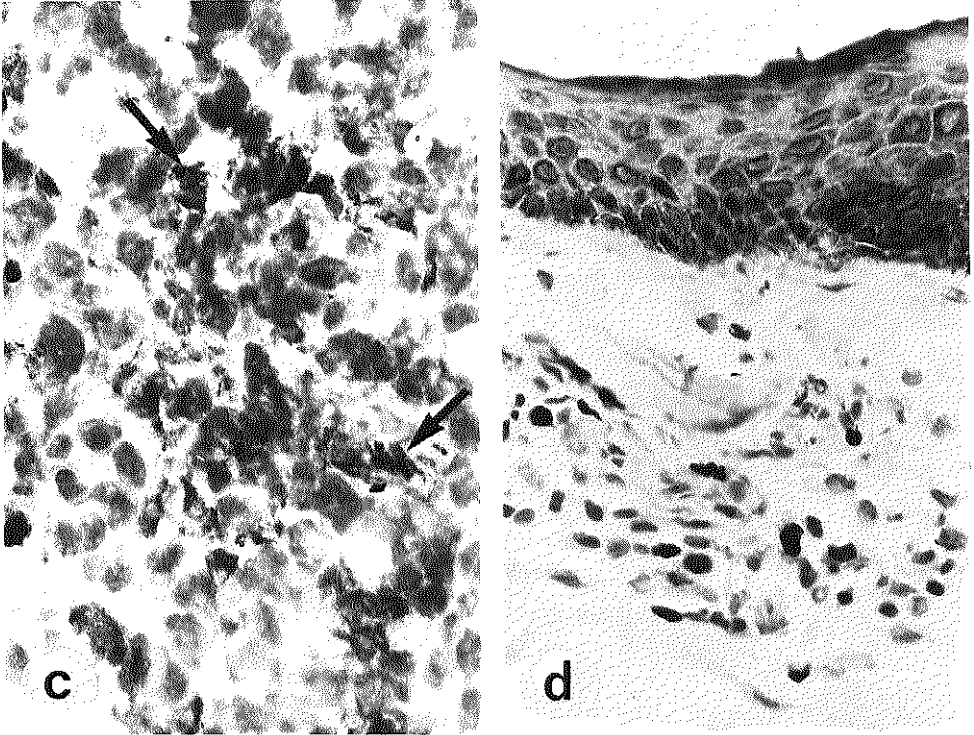
Figure 1c. The MAb JML-H14 specific for CD14 effectively stained Kupffer cells in the liver (arrow).
Figure 1d. In allogeneic skin biopsies, the MAb Ki67 reacted with the cytoplasm of the basal epithelial cells of epidermis and occasionally with the nuclei of the basal cell layer. Also the nuclei of several cells in the rejection infiltrate showed expression of the proliferation associated nuclear antigen (In all figures the magnification used is 300x).

E.4 Discussion

In this study it is confirmed that human specific monoclonal antibodies that are known to crossreact with their homologous counterpart in the rhesus monkey using indirect immunofluorescene staining techniques, immunoprecipitation studies or bioassays, are not *per sé* equally effective in immunohistochemical analysis of different tissues. More importantly, 23 MAbs are presented that can be used effectively for indirect

immunoperoxidase immunohistochemical studies in the rhesus monkey on frozen sections of lymph node, thymus, liver and skin. In this respect, a previous report by Sandusky et al [43] dealing with the reactivity of several human specific MAbs with different leucocyte markers in lymph nodes is extended with 20 other MAbs.

Approximately 60% of all MAbs with an 'excellent' target staining in FACS analysis, provided strong target marking and minor or no aspecific background coloring in frozen tissue sections using the indirect immunoperoxidase staining technique. Although a somewhat lower affinity of some MAbs in monkey tissues should be anticipated, the indirect conjugated peroxidase technique proved to be sufficiently sensitive for the visualization of antigen-antibody interaction in the majority of the MAbs tested. Thus, the need for a more sensitive immuno-histochemical staining technique, such as the avidin-biotin peroxidase complex method [22] is not indicated by this study.

The anti-human MAbs W6/32, anti-Leu10, and anti-HLA-DP which are known to be crossreactive with rhesus antigens using immunoprecipitation studies [37,8] could be used effective for immunohistochemical staining of target cells in lymphoid tissue. Remarkably, using anti-Leu10 and anti-HLA-DP no cells were positively stained in the allogeneic skin grafts. However, Genox 3.53, directed against the same molecule as anti-Leu10, did appear to stain lymphoid and stromal cells both in lymph node and the allogeneic skin. These observations might be an indication that also in the rhesus monkey, certain epitopes of the MHC class II antigens may not be exhibited constitutively. A similar phenomenon has been described with regard to the expression of HLA-DR (endothelial monocyte) antigens on B cells in man [7].

The two MAbs MD1 and 61E71, fully crossreacting with rhesus IFN-γ and TNF-α as demonstrated in a bioassay, both yielded very intense pericellular staining of small numbers of cells in lymph nodes. Similar observations were described for antibodies directed against IFN-γ in rejection infiltrates after kidney transplantation in the rat [42]. Therefore, it seems likely that MD1 and 61E71 demonstrate lymphokine producing cells in the rhesus monkey. A drawback of these MAbs was the presence of diffuse background staining in skin graft biopsies. This diffuse intercellular staining might be the result of free IFN-γ or free TNF-α.

The two MAbs FN18 and GM9, raised against rhesus CD3 and CD8 surface antigens respectively, indeed gave an intense specific staining reaction of lymphoid cells. However, FN18 also crossreacted with hair follicles in skin biopsies. Therefore, the MAb T11, human CD2 specific, might be more advantageous than FN18 as it fully crossreacted with the rhesus pan T cell population without staining irrelevant structures in lymph node, thymus or skin. Similar results for T11 and lymph node staining have been demonstrated previously [43]. In this elegant study, also reactivity of anti-Leu5b could be demonstrated with rhesus lymphoid cells. This could not be confirmed in our study. Possibly the use of more sensitive avidin

biotin technique by Sandusky et al. accounted for this discrepancy.

It was confirmed that anti-Leu6, CD1-specific very specifically stained mainly epidermal dendritic cells and also some dermal dendritic cells. Similar results have been described recently by Ringler et al. [40], in their paper on the reactivity of 28 MAbs specific for dendritic cells in the skin of the rhesus monkey.

Four out of the five MAbs of which crossreactivity had not been assessed previously, proved to be effective for immunohistochemical staining (see tables 4 and 5). Though further study is required to determine whether or not the same functional surface markers are stained, it can be stated that the MAbs specific for CD14, CD57 and a proliferation associated human nuclear antigen defined by Ki67, yielded labelling patterns that excellently matched the situation in man.

Firstly, this was the case for the majority of the CD14 specific MAbs that recognized macrophage-like cells in the lymph node and Kupffer cells in the liver (Table 4). In skin, histiocytes could be identified by light microscopy. However, the immunoperoxidase staining reaction with all MAbs, except for JML-H14, was faint and often accompanied by background staining. These observations suggest, that histiocytes in the nonhuman primate might exhibit similar specific morphologic and tissue-specific characteristics expressing distinct immunologic phenotypes as found in the human mononuclear phagocytic system [51].

Secondly, anti-Leu7, specific for CD57, expressed by T cell and Natural Killer cell subsets, proved to be the only MAb with a staining reaction in lymph node, though rather faint (Table 4). The Mab anti-Leu11b, specific for CD16 expressed by Natural Killer cells and neutrophils, did not show any crossreactivity with rhesus antigens in this study.

Thirdly, MAb Ki67, directed against the proliferation associated nuclear antigen, yielded a very clear nuclear staining reaction in all rhesus tissues. The staining pattern corresponded very well with human staining patterns, most notably in thymus. This result extends a previous report pointing out the evolutionary conservation in many mammalian species of this particular epitope [14]. Thus, it can be assumed that also in the rhesus monkey Ki67 stains cells that are not in G0 phase of the cell cycle. This MAb might be very functional for cell kinetic studies in the nonhuman primate.

In conclusion, we have demonstrated that membrane glycoproteins of different kinds of rhesus cells as well as a rhesus proliferation associated nuclear antigen can also be selectively identified immunohistochemically on frozen tissue sections by using anti-human monoclonal antibodies. We expect that the selected MAbs will be useful in studying cell interactions of the immune response in rhesus monkey models of human disease.

E.5 References

1.   Abo T, Balch CM: A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J immunol 127(3):1024-1029, 1981.
2.   Amhed-Ansari A, Brodie AR, Fultz PN, Anderson DC, Sell KW, McClure HM: Flow microfluorometric analysis of peripheral blood mononuclear cells from nonhuman primates: correlation of phenotype with immune function. Am J Primatology 17:107-131, 1989.
3.   Bakker NPM, Erck MG van, Zurcher C, Faaber P, Lemmens A, Hazenberg M, Bontrop RE, Jonker M. Experimental immune mediated arthritis in rhesus monkeys. A model for human rheumatoid arthritis? Rheumatol Int 10:21-29, 1990.
4.   Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, William AF, Ziegler A: Production of monoclonal antibodies to group A erythrocytes, HLA and other human surface antigens. New tools for genetic analysis. Cell 14:9-20, 1978.
5.   Bieber CP, Howard FD, Pennock J, Wong J, Shorthouse R and Stinson, EB: Preparation, characterization and primate testing of monoclonal antithymocyte globulin. Transplantation 31:283-289, 1981.
6.   Bogman MJJT, Dooper IMM, van de Winkel JGJ, Tax WJM, Hoitsma AJ, Assmann KJM, Ruiter DJ, Koene RAP: Diagnosis of renal allograft rejection by macrophage immunostaining with a CD14 monoclonal antibody, WT14. The Lancet ii:235-238, 1989.
7.   Bontrop RE, Claas FHJ, Baldwin W, Otting N, van Rood JJ, Giphart MJ. Molecular characterization of endothelial monocyte antigens. Immunobiology of HLA, Volume II, p585. New York, Springler-Verlag, 1989.
8.   Bontrop RE, Otting N, Elferink BG, Pham K, Broos LAM, de Vries RRP, Jonker M: Analysis of the chimpanzee MHC class II DR-subregion: a survey from gene to function. Hum Immunol 1990 (in press).
9.   Borleffs JCC, By-Aghai Z de, Marquet RL: Beneficial influence of Cyclosporine A and standard immunosuppression on kidney graft survival in transfused monkeys. Transplantation 32(2):161-162, 1981.
10. Brodsky FM, Parham P, Barnstable CJ, Crumpton MJ, Bodmer WF: Monoclonal antibodies for analysis of the HLA system. Immunological Rev. 47:3-61, 1979.
11. Chen Y, Evans RL, Pollack MS, Lanier LL, Phillips JH, Rousso C, Warner NL, Brodsky FM: Characterization and expression of the HLA-DC antigens defined by anti-Leu 10. Hum Immunol 10:221-235, 1984.
12. Daniel MD, Letvin NL, King NW, Kannagi M, Seghal PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC: Isolation of a T-cell tropic HTLV-II-like retrovirus from macaques. Science 288:1201-1204, 1985.
13. Dimitri-Bona A, Burmester GR, Waters SJ, Winchester RJ: Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 130:145-152, 1983.
14. Falini B, Flenghi L, Fagioli M, et al: Evolutionary conservation in various mammalian species of the human proliferation-associated epitope recognized by the Ki-67 monoclonal antibody. J Histochem Cytochem 37: 1471-1478, 1989.
15. Gadd S. Cluster report: CD14: In Leucocyte Typing IV, edited by Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr, p787. Oxford University Press, 1990.
16. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20, 1982.
17. Gerritsen WR, Wagemaker G, Jonker M, Kenter MJ, Wielenga JJ, Hale G, Waldmann H, Bekkum DW van: The repopulation capacity of bone marrow grafts following pretreatment with monoclonal

antibodies against T lymphocytes in rhesus monkeys. Transplantation 45:301-307, 1988.

18. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 68:932-941, 1981.

19. Haynes BF, Dowell DL, Hensley LL, Gore I, Metzgar RS: Human T cell antigen expression by primate T cells. Science 215:298-300, 1982.

20. Hogg N, MacDonald S, Slusarenko M, Beverly PCL: Monoclonal antibodies specific for human monocytes, granulocytes and endothelium. Immunol 53:753-767, 1984.

21. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA: A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J Immunol 126:2117-2122, 1981.

22. Hsu S, Raine L, Fanger H: A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am J Clin Pathol 75:734-738, 1981.

23. Jonker M, van Lambalgen R, Mitchell DJ, Durham SK, Steinmann L: Successful treatment of EAE in rhesus monkeys with MHC class II specific monoclonal antibodies. J Autoimm 1(5):399-414, 1988.

24. Jonker M, Van Meurs GJE: Monoclonal antibodies specific for B cells, cytotoxic/suppressor T cells, and a subset of cytotoxic/suppressor T cells in the rhesus monkey. In: Leucocyte Typing, edited by Bernhard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, p328. Berlin, Springer-Verlag, 1984.

25. Jonker M, Nooij FJM, Chatenoud L: Immunosuppression by monoclonal T cell antibodies. Transplant Proc 18(5):1287-1290, 1986.

26. Jonker M, Slingerland W: Reactivity of monoclonal antibodies specific for human CD markers with rhesus monkey leucocytes. In: Leucocyte Typing IV, edited by Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, vd Borne AEGKr, p1058. Oxford University Press; 1990.

27. Koning F, Fifth European Immunology meeting, 1982.

28. Koning F, Schreuder GMTh, Giphart MJ, Bruning JW: A mouse monoclonal antibody detecting a DR-related MT2-like specificity: serology and biochemistry. Hum Immunol 9(4):221-230, 1984.

29. Kung PC, Goldstein G, Reinherz EL, Schlossman SF: Monoclonal antibodies defining distinctive human T cell surface antigens. Science 206:347-349, 1979.

30. Lambalgen R van, Jonker M: Experimental allergic encephalitis in rhesus monkeys: I. Immunological parameters in EAE resistant and susceptible rhesus monkeys. Clin Exp Immunol 68(1):100-107, 1987.

31. Lampson LA, Levy R: Two populations of Ia-like molecules on a human B cell line. J Immunol 125(1):293-299, 1980.

32. Leeuwenberg JFM, Damme J van, Meager T, Jeunhomme TMAA, Buurman WA: Effects of tumor necrosis factor on the interferon-γ-induced histocompatibility complex class II antigen expression by human endothelial cells. Eur J Immunol 18:1469-1472, 1988.

33. Letvin NL, Daniel MD, Seghal PK, Desrosiers RC, Hunt RD, Waldron LM, MacKey JJ, Schmidt DK, Chalifoux LV, King NW: Introduction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science 230:71-73, 1985.

34. Letvin NL, King NW, Reinherz EL, Hunt RD, Lane H, Schlossman SF: T lymphocyte surface antigens in primates. Eur J Immunol 13(4):345-347, 1983.

35. Meide PH van der, Dubbeld M, Schellekens H: Monoclonal antibodies to human immune interferon and their use in a sensitive solid-phase ELISA. J Immunol Meth 79:293-305, 1985.

36. Nooij FJM, Borst JG, Meurs GJE van, Jonker M and Balner H: Differentiation antigens on rhesus monkey lymphocytes. I. Identification of T cells bearing CD3 and CD8, and a subset of CD8 bearing cells. Eur J Immunol 16:975-979, 1986.

37. Parham P, Seghal PK, Brodsky FM: Anti-HLA-A,B,C monoclonal antibodies with no alloantigenic

specificity in humans define polymorphisms in other primate species. Nature 279:639-641, 1979.

38.   Phillips JH, Babcock GF: NKP-15: a monoclonal antibody reactive against purifies human Naturel Killer cells and granulocytes. Immunol Letters 6:143-149, 1983.

39.   Rieber PJ, Lohmeyer J, Schendel DJ, Gottlinger H, Brodmann S, Rank G, Heydecke S, Kopp E, Riethmuller G: Characterization of functional human T cell subsets by monoclonal antibodies. In: Leukocyte Typing, edited by Bernard A, Bloumsell L, Dausset J, Milstein C, Schlossmann SF, p303. New York, Springer-Verlag, 1984.

40.   Ringler DJ, Hancock WW, King NW, Murphy GF: Characterization of nonhuman primate epidermal and dermal dendritic cells with monoclonal antibodies. Lab Invest 56(3):313-320, 1987.

41.   Roger JH, Vreeswijk W van, Balner H: The MHC of rhesus monkeys: XII. Current knowledge of DR and other B-cell specific antigens. J Immunogenet 7:333-347, 1980.

42.   Ruers TJM, Buurman WA, Boxtel CJ van, Linden CJ van der, Kootstra G: Immunological observations in rat kidney allografts after local steroid administration. J Exp Med 166:1205-1220, 1987.

43.   Sandusky GE, Horton PJ, Wightman KA: Use of monoclonal antibodies to human lymphocytes to identify lymphocyte subsets in lymph nodes of the rhesus monkey and the dog. J Med Primatol 15:441-451, 1986.

44.   Soulilliou JP, Mauff B le, Cantarovich D, Giral M, Hourmant M, Jacques Y: Anti-IL-2-R monoclonal antibody in allograft recipients. J Autoimm 1:655-661, 1988.

45.   Stevens HPJD, Hovius SER, Vuzevski VD, Nierop PWM van, Gotte M, Roche NA and Jonker M: Immunological aspects of allogeneic partial hand transplantation in the rhesus monkey. Transpl Proc 22(4):2006-2008, 1990.

46.   Stevens HPJD, Kwast ThH van der, Meide PH van der, Buurman WA, Jonker M: Synergistic immunosuppressive effects of monoclonal antibodies specific for interferon gamma and tumor necrosis factor alpha. Transplantation 50(5):856-861, 1990.

47.   Stevens HPJD, Roche N, Hovius SER, Jonker M: In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive cells. Transpl Proc 22(4):1783-1784, 1990.

48.   Urdal DL, March CJ, Gillis S, Larsen A, Dower SK: Purification and chemical characterization of the receptor for interleukin 2 from activated human T lymphocytes and from a human T-cell lymphoma cell line. Proc Nath Acad Sci USA 81:6481-6485, 1984.

49.   Vreeswijk W van, Roger JH, D'Amaro J, Balner H: The major histocompatibility com-plex of rhesus monkeys: VII. The identification of five new serological defined antigens. Tissue Antigens 9:17-30, 1977.

50.   Watson AJ, DeMars R, Trowbridge IS, Bach FH: Detection of a novel human class II HLA antigen. Nature 304:358-361, 1983.

51.   Wood GS, Turner RR, Shiurba RA, Eng L, Warnke RA: Human dendritic cells and macrophages. In situ immunophenotypic definition of subsets that exhibit specific morphologic and microenvironmental characteristics. Am J Path 119(1):73-82, 1985.

52.   Wood GS, Warner NL, Warnke RA: Anti-Leu3/T4 antibodies react with cells of monocyte/macrophage and Langerhans Lineage. J Immunol 131:212-216, 1983.

CHAPTER III: PARTIAL HAND TRANSPLANTATION
STUDY A : Technical aspects

S.E.R. Hovius & H.P.J.D. Stevens

A.1 Introduction

The way to allogeneic transplantation of extremities seems to be open, for the immunosuppressive agent Cyclosporin A (CyA) has made it possible to obtain long term graft survival of clinical allotransplantations of kidney[1], heart[2], liver[3] and other organs[4, 5]. Furthermore, experimental and clinical allotransplantation of bone and joints[6], muscle[7], nerve[8] and skin[9] has been performed with promising results[10]. Experimental transplantation of a composite of these tissues (i.e. the limb), utilizing CyA, has been performed in the rat by the groups of Furnas[11], Fritz[12] and Kim et al[13]. Prolonged survival of limb allografts treated with CyA could be established even across a strong mismatch, i.e. a major histocompatibility (MHC) border. In the non human primate, transplantation of the hand has only been performed by Daniel et al in 1986[14,15] and Stark et al in 1987[16,17]. Total hand transplants were performed by these groups in baboons under continuous, high dose CyA and Prednisone treatment. Graft survival longer than 70 days, was achieved in two out of four and one out of eight cases, respectively. Both studies showed that recovery of motor and sensory function after hand transplantation could be achieved. In our studies the radial side of the hand was transplanted in a rhesus monkey model since the anatomy and functional use of the hand of the monkey is closely related to the human hand. The radial side of the hand (the first ray) is a composite tissue unit in which sensory and motor function recovery can be evaluated. Transplantation of only the radial side of the hand is also more ethical since basic hand function can still be maintained in the monkey. The rhesus immune system, including MHC antigens, shows great similarity with the human immune system. Therefore results obtained in this research model allow for better extrapolation to the situation in the human being.

General practicability of the research model and functional recovery has been demonstrated in an autologous (replant) situation, previously. To determine the value of monoclonal antibodies (mAbs) for reversal of rejection episodes of the radial hand, a combination of mAbs (specific for several surface markers on immunocompetent cells) was developed in skin transplantation studies[18]. Furthermore preoperative third party blood transfusions are known to exert a protective effect on organ allograft survival[19,20]. The value of blood transfusions in non human primate composite tissue transplantation has yet to be determined, for this reason they were added to our study. Laser Doppler flowmetry (LDF) and temperature recordings have demonstrated their beneficial use with regard to monitoring of the microcirculation after microvascular surgery[21,22]. These devices were used for monitoring of the microcirculation in the transplant model.

This paper concerns the feasibility of allogeneic transplantation of the radial side of the

hand, the value of monitoring of the microcirculation and a preliminary report on functional recovery in an allogeneic model in the rhesus monkey.

A.2   Material and methods
A.2.1 Animals

Rhesus monkeys (Macaca Mulatta) were born and raised in the TNO Primate Center, Rijswijk, the Netherlands, employing a "harem type" breeding system. Average weight was 7.5 kg, age varied from nine to 24 years and male to female ratio was two to one. The animals were selected on absence of prior alloimmunization, on normal kidney and liver functions and non-injured, relatively large hands. If possible left or right handedness was assessed.

Animal care complied with the "Principles of Laboratory Animal Care" and the "Guide for the care and use of laboratory animals" (NIH publication no. 80-23, revised 1978).

A.2.2 Experimental groups and immunosuppressive therapy

The protocol, designed in cooperation with the department of biostatistics, consisted of twelve successful allogeneic transplantations of the radial side of the hand in the rhesus monkey. Transplantations were performed in unrelated donor-recipient combinations, mismatched for rhesus major histocompatibility (RhLA) -A, -B and DR-antigens. A group of twelve monkeys was divided at random into four different treatment subgroups with three monkeys per subgroup in a statistical factorial design according to Fisher[23] (table I). In this scheme the effects of two factors alone and in combination could be determined in the same study. These factors were preoperative third party blood transfusions and monoclonal antibodies (mAbs) for the treatment of rejection episodes.

All recipients received immunosuppressive treatment consisting of Cyclosporine A (CyA) and Prednisone (DiadresonFaquaosum = DAF). CyA was administered by subcutaneous injections twice daily (25 mg/kg/d, dissolved in Miglyol 812 and absolute ethanol as per Sandoz), to obtain whole blood trough levels of 400 - 1000 ng/ml[24]. DAF was given in an initial high dosage (12 mg/kg/day) for the first three days postoperatively and then was tapered slowly until a maintenance dose (1 mg/kg/day) was reached twelve days later.

Following the protocol in table 1, six monkeys received three third party blood transfusions, consisting of 20 ml. of fresh whole citrated blood from random donors, completely

mismatched for the transplant recipient and donor RhLA antigens. Blood transfusions were given at biweekly intervals (-6, -4 and -2 weeks) before transplantation.

Onset of rejection was defined as the first obvious sign of swelling, erythema and blister formation of the transplant together with histological confirmation for the presence of rejection infiltrate, edema, hemorraghes and epidermiolysis. In case of rejection six monkeys were treated with an increase in DAF to the initial dosage (12 mg/kg/d), followed by the same tapering scheme as mentioned before. In the six, rejection was treated with a combination of mAbs, specific for different surface antigens on immunocompetent cells administered as an intravenous bolus injection for a period of ten days[25].

A.2.3 Anesthesia, medication and monitoring

Apart from routine general anesthesia with standard monitoring of physiological parameters a continuous axillary plexus block was administered (mepivacaine and adrenaline, 20 ml 1% and 0.1 mg respectively, per eight hours). An intravenous infusion of Dextran 40 (Rheomacrodex, 1.5 gr/kg) was given during and directly after operation for hemodilution. Cefamandol (R/Mandol, 100 mg/kg/d in two dosages) was administered intramuscularly for five days starting an half hour before operation as antibiotic prophylaxis. Acetylsalicylic acid (R/Aspegic, one dosage of 100 mg) was given intramuscularly on the day of operation to decrease platelet aggregation.

The microcirculation was monitored during and directly after surgery with temperature recordings of the transplant compared to a control temperature of the ipsilateral middle finger. Also Laser Doppler flowmetry (R/Perimed, PF2, Sweden) was used to measure blood flow in the microcirculation of the skin of the transplant[21]. Measurements were performed after dissection at the donor site with the transplant on its vascular pedicle and after reanastomizing of the vascular pedicle at the recipient site. The monkeys were kept tranquilized for the first 4-6 hours after surgery for general and microcirculatory monitoring. After this period appropriate dressings and a custom made thermoplastic splint were applied to protect the operated upper extremity[26].

A.2.4 Operation

Before operation the donor and recipient arm were shaved. After general anaesthesia the donor rhesus monkey was placed in lateral position with the arm abducted.

The transplant, consisting of the first ray of the hand enlarged with the radial forearm flap (for monitoring and biopsies, size approximately 20 cm^2)[27] was marked (see fig. 1). All operations were performed under sterile conditions using tourniquet control. In the forearm, the radial forearm flap was dissected. The flexor, extensor and abductor pollicis longus muscles were cut at the wrist and marked, the adductor muscle was freed from its origin. The median nerve, the palmar cutaneous branch of the median nerve, the motor branch of the ulnar nerve beneath the adductor muscle and the superficial radial nerve branch to the thumb were identified, dissected, cut proximally and separately marked. The cephalic vein and the radial artery were the vascular pedicle to the transplant. They were dissected proximal to the radial forearm flap. An osteotomy was performed at the base of the first metacarpal bone. The tourniquet was freed at the upper arm after careful hemostasis by bipolar

Figure 1. The transplant model. The dotted line marks the radial hand unit. Ventral: A = adductor muscle; T = thenar muscle; U.n. = ulnar nerve; M.n. = median nerve; f.p.l. = flexor pollicis longus; a.p.l. = abductor pollicis longus; r.a. = radial artery. Dorsal: e.p.l. = extensor pollicis longus; a.p.l. = abductor pollicis longus; s.r.n. = superficial radial nerve; c.v. = cephalic vein.

coagulation, the transplant was monitored with LDF and temperaturerecordings. During dissection of the donor site the recipient monkey was given general anaesthesia and an axillary plexus block on another operating table. Skin incisions were performed in the same manner as the donor site and adjusted for size discrepancies. The same nerves and tendons were identified and marked. They were cut distally to adjust for size difference and to allow nerve anastomoses without tension. The recipient's thumb was amputated proximally at the first metacarpal bone, with thenar muscles and adductor muscle. After tourniquet release hemostasis was ensured. Following transection of the vascular pedicle of the transplant at the
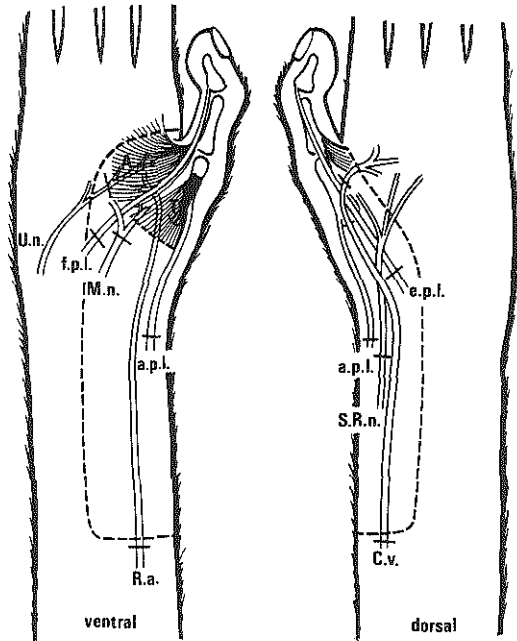
donor site, the defect was reduced by excision of the trapezium and covered with a full thickness graft from the belly.

After receipt of the donor transplant at the recipient site (fig. 2a and 2b), osteosynthesis was performed at the donor and recipient metacarpal bone with transosseus wiring. The adductor muscle of the transplant was reinserted at the third metacarpal bone. Subsequently the nerve ends of the motor branch of the ulnar nerve, the common trunk of the median nerve, the palmar cutaneous nerve and the superficial radial nerve were anastomosed with 10x0 nylon sutures (BV 6, R/Ethilon) under the microscope after adjustment for length. The tendons of the flexor pollicis longus, the extensor pollicis and abductor pollicis longus were sutured with 5x0 nylon. Recipient radial artery and cephalic vein were anastomosed with their donor counter parts end-to-end with 10x0 nylon sutures (BV-6, R/Ethilon) under the microscope. Before the vessels were sutured the transplant artery was flushed with dextran 40 - heparin solution to rinse the transplant from donor serum and blood cells as much as possible. The skin was loosely approximated and drains were applied. After release of the small vessel clamps the transplant was monitored immediately with a pinprick test for bleeding as well as temperature and Laser Doppler flowmetry recording.

A.2.5 Postoperative care

Wound inspection, assessment of bodyweight and central temperature were carried out regularly. After removal of the splint six to nine weeks postoperatively wound inspection could be performed daily.

The microcirculation of the flap was monitored in the same frequency as mentioned above, by using the pin-prick test, capillary refill assessment if possible and temperature recordings of the flap and pulp of the thumb as well as the pulp of the middle finger. Temperature recordings were always done in the same manner in a controlled temperature environment. LDF of the forearm flap was done by placing the probe holder on a marked spot and measuring four independent values for the duration of one minute. To determine the predictive value of LDF measurements with regard to onset of rejection, trends over one week and over two weeks before starting anti-rejection therapy were evaluated. For statistical analysis a minimum of two measurements in the first seven or four in the first fourteen days prior to onset of anti-rejection therapy were evaluated.

All tests (laboratory, immunological and histology tests) were taken regularly and in case of rejection the frequency of testing was increased.

Laboratory tests included red and white blood cell counts as well as assessment of levels

of electrolytes and kidney and liver function.

Immunological tests consisted of assessment of CyA whole blood trough levels, with a specific radioimmunoassay (RIA, Sandimmun-kit, Sandoz Ltd., Basle, Switzerland). Further immunological details on tests and results are presented elsewhere[25].

Regularly, full thickness biopsy punches (diameter 3 mm) were taken. The biopsy was fixated with formalin 4% and processed routinely for hematoxylin-azofloxine analysis. Histological findings were scored semiquantitatively under light microscopy.

A.2.6 Sensory and motor function recovery

To evaluate sensory nerve regeneration, the sensitivity to noxious stimulation was assessed weekly by means of a small electric current (1.8 milliAmpere) applied locally to the skin of the conscious monkey to provoke a withdrawal reflex. The number of sites rendering positive (withdrawal) reactions in relation to the total number of measuring sites gives the percentage of sensory reinnervation.

Motor reinnervation was evaluated by detection of Compound Motor Action Potentials (CMAP) by electromyography of the thenar muscles after ruling out inadvertent stimulation of the ulnar nerve. Temperature corrected amplitude of CMAP of the abductor pollicis brevis muscle in the transplanted and contra-lateral side were compared weekly starting immediately postoperatively. Further details and histology concerning reinnervation will be provided elsewhere[28].

A.2.7 Statistical analysis

When appropriate, Fisher's exact-test, Mann-Whitney U test, two sample t-test, single regression analysis and log-rank test were performed for hypothesis testing. Differences were considered significant if $P < 0.05$.

A.3 Results
A.3.1 Experimental groups

In a consecutive series 14 transplantations of the radial side of the hand were performed, of which 12 were successful. Two technical failures, due to vascular occlusion were excluded.

Graft survival times were short in six cases (21-33 days) and long in the other six (79-179 days, see table 1 and fig. 2c and 2d). CyA whole blood trough levels were above the minimal required doses of 400 ng/ml[24] in 83% of all cases. In the long term survival group the mean CyA level was 1025 (SD=193) ng/ml, with 86% above the therapeutic margin. In the animals where the allografts were rejected before day 33, 74% of all postoperative CyA levels were in the therapeutic range and of the CyA levels after the 5th postoperative day even 92%. Long term and short term survivors showed no significant difference between CyA levels during day 5 and start of anti-rejection therapy (P>0.05, Mann-Whitney U). There was no significant correlation between allograft survival times and CyA levels during the experiment (P>0.05, regression analysis).

Between the two groups of animals that did or did not receive preoperative third party blood transfusions, no significant difference in the onset of allograft rejection could be observed, nor did it facilitate reversal of graft rejection (P>0.05, log-rank).

Table 1. Allogeneic partial hand transplantation in 12 rhesus monkeys
under cyclosporine A (CyA) and prednisone (DAF)

Protocol[a]	Monkey no.	Start of rejection therapy (days)	Rejection reversed	Graft survival time (days)
I(+,+)	2799	79	-	79[b]
	4023	62	+	
		111	+	121[b]
	2988	15	-	22[b]
II (+,-)	3992	139	-	144
	3439	41	+	
		75	+, partial	97[b]
	3308		-	179[b]
III (-,+)	2596	13	-	30
	2I	21	-	29[b]
	3310		-	85[b]
IV (-,-)	1FU	21	-	33
	1550	21	-	33
	3212	12	-	21

[a]Protocol: I(+,+) = cocktail of monoclonal antibodies during rejection (mAbs), pre-operative third party blood transfusions (transf.); II (+,-) = mAbs, no transf; III (-,+) = raise in DAF during rejection, transf; IV (-,-) = DAF, no transf.
[b]death of rhesus monkey (spontaneously or due to euthanasia).
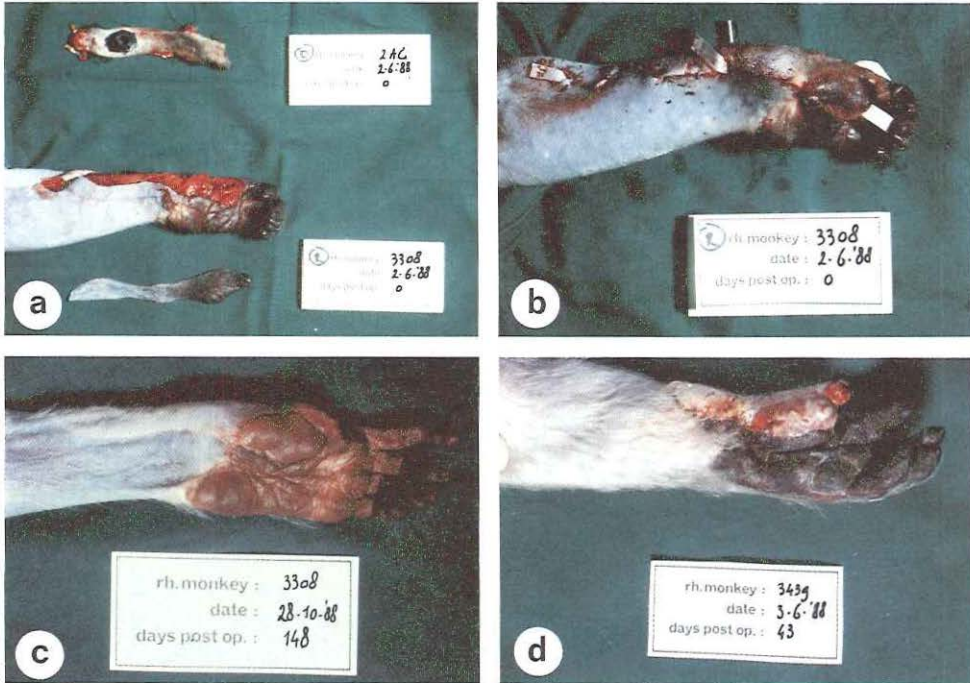
Figure 2a. Arm of recipient rhesus monkey (#3308) at operation. The radial unit of donor rhesus monkey (#2AC) is shown at the top of the photograph just before transplantation. The lower recipient thumb is discarded.

Figure 2b. After completion of the radial hand transplantation (#2AC to #3308).

Figure 2c. Rhesus monkey (#3308) 148 days after transplantation. Normale skin texture with normal appearance of the allograft.

Figure 2d. Example of a transplant rejection in a rhesus monkey (#3439) 43 days after transplantation. Note the swelling and skin slough.

Swelling, dermal hemorrhages, epidermolysis of the allograft, histologically confirmed to be rejection, occurred in ten out of 12 recipients (table 1). Histologically slight to severe lymphohistiocytic infiltrates, edema, hemorraghies and epidermolysis were seen (fig. 3a). In all transplants skin was more rapidly rejected than nerve and muscle. Five monkeys received the combination of mAbs for treatment of the rejection episode. In two out of five cases, graft rejection could be reversed (table 1, fig. 3b).
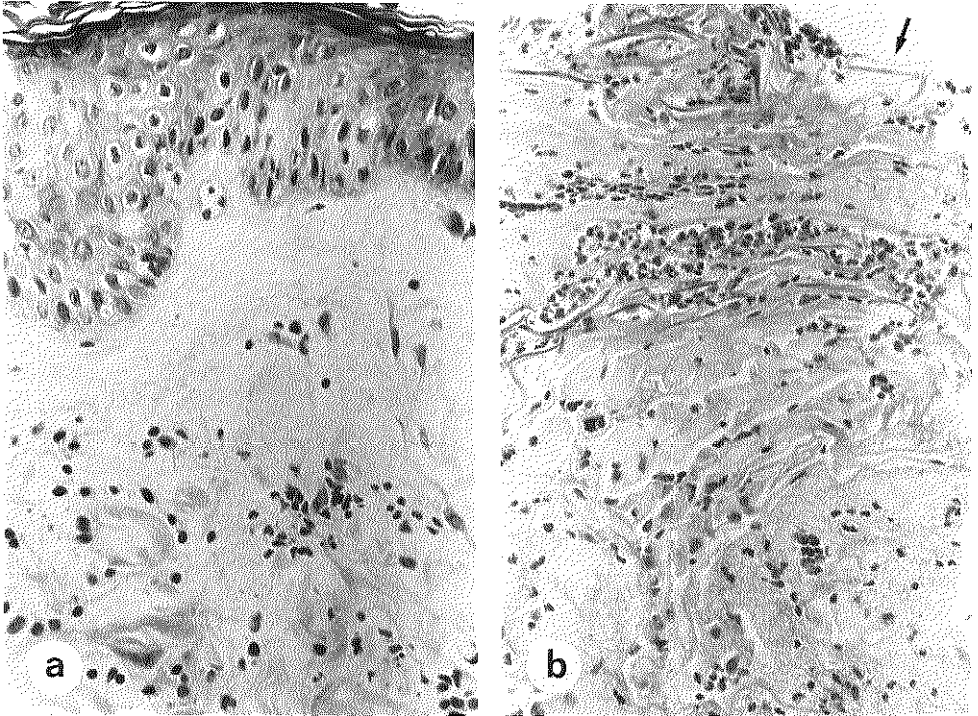
Figure 3a. A H&A stained section of the allograft of monkey 3439; 43 days postoperatively, two days after the onset of MAbs anti-rejection therapy. The immune response of the host has resulted in total loss of the skin of the allograft (arrow), hemorraghe and also a diffuse infiltrate of lymphocytes and macrophages is visible (magnification 300x).
Figure 3b. A H&A stained section of the allograft of the same monkey (3439), 47 days postoperatively, after seven days of anti-rejection therapy with MAbs. Reversal of rejection has allowed for regeneration of the epidermis. The number of infiltrated cells has greatly diminished (magnification 300x).

In these monkeys also a second rejection episode could be treated successfully. In the other five partial hand recipients, graft rejection was treated by increasing the doses of DAF. In none of these cases, could graft rejection be reversed. MAb anti-rejection therapy prolonged graft survival significantly longer than an increase in steroids (P=0,015, log-rank). Furthermore, histological severity of rejection at onset of anti-rejection therapy did not influence outcome of rejection treatment (P > 0.05).

A.3.2 Anesthesia and operation

No complications of anesthesia were encountered. Operating time varied from 5 to 9 hours.

A.3.3 Postoperative care

Postoperative pin-prick test in the pulp of the thumb showed instant bright red blood drops in most cases. Regarding temperature recordings one technical failure was already indicated in retrospect by temperatures of 28-29° Celsius of the thumb directly after operation. In rejection cases with necrosis of the thumb temperatures were below 30° Celsius. In all other cases where the transplants were viable, temperatures were above 30° Celsius. In the two technical failures LDF measurements were below 10 perfusion units three days after operation. To determine the predictive value of LDF measurements with regard to onset of rejection, trends over seven and over fourteen days prior to onset of rejection, were evaluated. Values measured over one week, nor over two weeks prior to therapy allowed any conclusion.

At onset of anti-rejection therapy allograft infiltration ranged from slight to severe diffuse. In the two monkeys with mAbs therapy (4023 and 3439) where reversal of rejection was established, already after 4-7 days of treatment only a slight focal infiltrate remained. In monkeys where rejection was not reversible graft rejection was complete in 4-10 days after onset of therapy, except for one monkey (2I) that died eight days after anti-rejection treatment.

A.3.4 Sensory and motor function recovery

In the six monkeys with short graft survival times (21 - 33 days) rejection occurred before sensory recovery could be shown. In the six monkeys with long term graft survival (79 - 179 days) first signs of sensory recovery appeared in the median nerve distribution after a mean of 42 days postoperatively (range 27-64 days). As soon as the first sign of sensory recovery was detected and reconfirmed, the custom made splint was no longer applied (mean : 38.7 days; range 32-57 days)(table II).

The percentage of median nerve reinnervation increased in time, episodes of rejection almost immediately decreased this area. In case of reversal of allograft rejection renewed

reinnervation was seen, within two weeks after the initial decrease (or initial absence) of reinnervation.

In six monkeys first signs of motor recovery could be assessed at a mean of 43,8 days postoperatively (range: 31-56 days).

In two out of six monkeys with short term graft survival (21 - 33 days) thenar muscle reinnervation could be detected even after onset of rejection. The quality of conduction

Table 2. Sensory and motor function recovery after allogeneic partial hand
transplantation in the rhesus monkey

Monkey	Start of rejection therapy (days)	First sign of of sensory (days)	Maximal percentage of median nerve reinnervation[a]	First sign of motor recovery (days)
2799	79	27	75	72[b]
4023	62	41	91	23
	111			
3992	139	35	80	79[b]
3439	41	56	14	37
	75			
3308	-	64	100	25
2I	21	-	-	22
3310	-	29	84	28
IFU	21	-	-	33

[a]The percentage of median nerve reinnervation is calculated by dividing the number of
positive measurements through the total number of measurement sites on the skin supplied
by the median nerve.
[b]First measurement by electromyograph.

(amplitude of CMAP) as well as the velocity of conduction (latency of CMAP) increased in time. More detailed information on sensory and motor function recovery is described elsewhere[28].

A.3.5 Complications

During immunosuppressive therapy overall weight loss was 13.4% in relation to preoperative weight. In the six long term survivors weight loss was observed ranging from 8 to 40% with an average of 22.4%.

Elevated levels of creatinine occurred in five out of twelve cases. Clearly elevated levels of lactate dehydrogenase and aspartate transaminase were observed in three cases, in four cases liver function parameters were incidentally elevated.

In four monkeys lymphoid tumor was found of which three died due to cachexia and tumor growth. In the first, a histiocytic sarcoma (2988) occurred 22 days after operation, in the second a malignant lymphoma (B cell type, 3439) 97 days after treatment with CyA, DAF and mAbs and two rejection episodes, and a histiocytic and fibrocytic sarcoma 179 days after operation without occurrence of rejection in the third monkey.

Three monkeys died of sepsis. One of these three (4023) died of multiple organ failure and sepsis after treatment with CyA, DAF and mAbs and two rejection episodes. There was evidence of tumor development in lymphoid tissue in this monkey. Of the other two one died of organ failure and sepsis after 29 days (2I) and the other (3310) died of sepsis presumably from the skin with dissemination to the central nervous system. One monkey (2799) died of shock and irreversible coma after administration of the first intravenous dosage of the mAbs cocktail. MAbs therapy did not have a significant effect on death in general $(P > 0.05)$[29].

A.4 Discussion

Long term survival combined with functional recovery is obligatory before allogeneic transplantation of extremities is considered. Experimental allogeneic composite tissue transplantation has long been focussed on the improvement of survival[6,11,12,13]. More recently, functional recovery has been under investigation[14,17,30]. This study was designed to investigate survival as well as functional recovery of a composite tissue allograft in the nonhuman primate.

To suppress the host-immune response against composite tissue allografts the combination of continuous high dosage of CyA (20-25 mg/kg/day) and prednisone is needed to establish long term allograft survival. This is demonstrated in various studies in rat[11,13] as well as monkey[14,17,24].

In the present study the same regime was chosen. Although CyA levels were above the therapeutic margin of 400 mg/ml still ten out of twelve hand transplantations showed rejec-

tion.

Mean skin graft survival times without immunosuppressive treatment are normally not longer than ten days[18,31]. In the short term survival group of this study graft survival time varied in six rhesus monkeys from 21 - 33 days. As the earliest rejection started much longer than ten days following operation unresponsiveness to CyA is unlikely. Furthermore, retrospective analysis ruled out a possible negative influence on allograft survival in female recipients, due to prior sensitization.

Onset of rejection was treated in our study either with an increase in DAF or with the mAbs therapy. Daniel et al.[14] and Stark et al.[16] reported reversal of rejection with an increase in prednisone in two out of four and one out of eight hand transplantations in baboons respectively. However, in their studies still two out of three showed skin problems after reversal. In our project, all radial hand transplants treated with an increase in steroids in case of rejection, did not show any signs of reversal. Regarding the use of mAbs in the treatment of acute graft rejection the beneficial use was first demonstrated by Cosimi et al in 1981[32] in the treatment of a rejection crisis following kidney transplantation. This has been reconfirmed by others[33]. In our study, mAbs therapy could reverse graft rejection in two out of five monkeys, on two occasions. Even though two out of the three other monkeys in this group died during mAbs anti-rejection therapy before the allograft was completely rejected, graft survival could be prolonged significantly longer than an increase in steroids. Data concerning lymphocytic subset distributions in the peripheral blood as well as in the allograft itself is presented elsewhere[25].

Third party and donor-specific blood transfusions have been proven to exert a protective effect on allograft survival, even under CyA therapy[20,34,35]. However in our study third party blood transfusions did not postpone the onset of graft rejection significantly, nor did they facilitate reversal of graft rejection. Possibly the high dosage of CyA masks the effect of blood transfusions.

Monitoring of microvascular surgery, as for instance LDF and temperature recordings, can be a helpful tool in detecting vascular compromise. Particularly if monitoring is performed continuously. As far as technical failures are concerned, one case could not be predicted as it occurred two days after surgery, the other had in retrospect low monitoring values directly after operation.

Black et al.[36] have demonstrated that a decline of the muscle temperature proved to be a reliable quantitative determination of limb allograft survival. In our study skin temperature recordings could not predict a rejection episode. Only skin necrosis produced a drop in skin temperature below 30° Celsius. Probably muscle temperature is less influenced by rejection or environment than skin temperature. To determine a predictive value of LDF

measurements with regard to onset of rejection very frequent measurements are needed without too much variability between measuring sessions. No conclusions could be drawn in this respect due to the variability of the values and the inability to measure caged monkeys very often. In allogeneic limb transplantation in rats where LDF values were less variable and more frequently measured, they correlated well with graft rejection although a predictive value could not be established[22].

Sensory and motor recovery occurred in all long term survivors. First sign of sensory recovery, assessed in conscious monkeys, was found after a mean of 41.8 days postoperatively (range: 27-64 days). Episodes of rejection almost immediately decreased the area of sensory recovery. As sensory receptors are localized in the dermal-epidermal junction they are bound to be affected as this area appears to be the main target of rejection[37-39]. Reversal of graft rejection enabled renewed reinnervation within two weeks after the initial decrease of reinnervation, thus host axons can reestablish reinnervation after rejection in allogeneic tissue.

First signs of motor reinnervation appeared after a mean of 43.8 days postoperatively (range: 31-56 days). In both monkeys in which reversal of rejection was demonstrated, also a negative effect on latency and amplitude of the compound motor action potential was observed[28]. A decrease in motor and sensory function after rejection, have also been described by others[14,38].

Side effects of the immunosuppressive therapy in this study mainly concerned weight loss due to anorexia in the long term survivors. Though aberrant biochemical values were present, no severe nephro- and hepatotoxicity were observed. However, if similar high doses are administered in clinical transplantation, more severe kidney and liver damage are likely to occur[10,40]. As three monkeys died of the consequences of lymphoid tumor development and three due to opportunistic bacterial infections the immunosuppressive treatment might have been too aggressive although similar doses of CyA and DAF were administered in baboons with hand transplants for longer periods without such side effects[14,16]. A possible reason could be that the baboons were young, jungle caught without previous history in contrast to older, inbred rhesus monkeys with a case history, varying from kidney donorship to testis radiation.

In conclusion, technical feasibility of allogeneic transplantation of the radial side of the hand in the rhesus monkey has been demonstrated. Graft survival was never longer than 180 days after surgery even under high dosages of Cyclosporine A and prednisone.

Reversal of graft rejection could be established twice in two out of five cases with monoclonal antibodies anti-rejection therapy and resulted in a significant prolongation of allograft survival times. An increase in DAF as anti-rejection therapy could not prevent

further graft rejection.

A predictive value for the onset of rejection could not be assessed by laser doppler flowmetry, due to variability of measurements and inability to measure more often.

Sensory and motor function recovery occurred in all long term survivors. Rejection had a negative influence on sensory and motor function recovery. Reversal of rejection enabled renewed sensory reinnervation within two weeks after anti-rejection therapy was started.

A more effective immunosuppressive regime is needed before actual allogeneic transplantation of the human hand can be performed.

A.5 References

1. Tilney N.L., Milford E.L., Carpenter C.B. et al. Long-Term Results of Cyclosporine Treatment in Renal Transplantation. Transpl. Proc., Vol. XVIII, no. 2, Suppl. 1 (April), pp 179 - 185, 1986.
2. Reichenspurner H., Odell J.A., Cooper D.K.C. et al. Twenty Years of Heart Transplantation at Groote Schuur Hospital. J. Heart Transplantation no. 6, pp. 17-23, 1987.
3. Gordon R.D., Shaw B.W., Iwatsu K.I.S. et al. Sandimmun' (SIM) in liver transplantation: indications for transplantation and survival. Surg.Clin.N.Amer. 66, pp 541-556, 1986.
4. Forman S.J., Blume K.G., Krance R.A. et al. Sandimmun' (SIM) vs. methotrexate (MTX) for prevention of graft vs. host disease after bone marrow transplantation.
Transpl. Proc. 19, no. 1 (Book III), pp 2605-2607, 1987.
5. Cohen Z., Silverman R.E., Wassef R. et al. Small intestinal transplantation using Sandimmun' (SIM). Transplantation 42, pp 613-621, 1986.
6. Doi K., DeSantis G., Singer D.I. et al. The effect of immunosuppression on vascularised allografts. Journ. of Bone and Joint Surg. vol. 71B, no. 4, pp 576-582, 1989.
7. Gulati A.K., Zalewski A.A. Muscle allograft survival after Cyclosporin A immunosuppression. Exp. Neurol. 77: 378-385, 1982.
8. Bain J.R., Mackinnon S.E., Hudson A.R. et al. Preliminary Report of Peripheral Nerve Allografting in Primates Immunosuppressed with Cyclosporine A. Transpl. Proc., vol 21, no. 1 (February), 1989: pp 3176-3177.
9. Hewitt Ch.W., Black K.S., Aguinaldo A.M.A. et al. Cyclosporine and skin allografts for the treatment of thermal injury. Transplantation, vol. 45, no. 1, pp 8-12, 1988.
10. Green C.J. Experimental Transplantation and Cyclosporine. Transplantation, vo. 46, Suppl (August), pp. 3-10, 1988.
11. Furnas D.W., Black K.S., Hewitt C.W. et al. Cyclosporine and Long-term Survival of Composite Tissue Allografts (Limb Transplants) in Rats. (With historical notes on the role of Plastic Surgeons in Allotransplantation.) Transpl. Proc., vol. XV, no. 4 suppl. 1: 3063-3068, 1983.
12. Fritz W.D., Swartz W.M., Rose S. et al. Limb allografts in rats immunosuppressed with Cyclosporin A. Ann. of Surgery, vol. 199, no. 2: 211-215, 1984.
13. Kim S.K., Aziz S., Oyer Ph. and Hentz V.R. Use of Cyclosporin A in Allotransplantation of Rat Limbs. Ann. of Plastic Surg. Vol. 12, no. 3: 249-255, 1984.
14. Daniel R.K., Egerszegi E.P., Samulack D.D. et al. Tissue transplants in primates for upper extremity reconstruction: A preliminary report. Journ. of Hand Surgery, Am. vol. 11a, no. 1: 1-8, 1986.

15. Egerszegi E.P., Samulack D.D., Daniel R.K. Experimental models in Primates for Reconstructive Surgery utilizing Tissue Transplants. Ann. of Plastic Surgery, vol. 13, no. 5: 423-430, 1984.
16. Stark G.B., Swartz W.M., Narayanan K. et al. Hand Transplantation in Baboons.
    Transpl. Proc..vol. 19, no. 5: 3968-3971, 1987.
17. Stark G.B., Swartz W.M., Narayanan K. et al. Allograft Composite Tissue Transplantation: Experimental Hand Transplantation in Primates. In: E. Riediger & M. Ehrenfeld (Eds.): Microsurgical Tissue Transplantation. Quintessenz-Verlag, Munich, 1989.
18. Stevens H.P.J.D., Roche N., Vuzevski V.D, Hovius S.E.R., Jonker M. In vivo additive immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II antigens. Transpl. Proc., vol. 22 (4): 1783 - 1784, 1990.
19. Opelz G. and Terasaki P.I. Importance of preoperative (not peroperative) transfusions for cadaver kidney transplants. Transplantation, 32, pp. 161, 1981.
20. Melzer J.S., Husing R.M., Feduska N.J. et al. The beneficial effect of Pretransplant blood transfusions in Cyclosporine-treated cadaver renal allograft recipients. Transplantation, vol. 43, no. 1, p. 61-64, 1987.
21. Heden P.G., Hamilton R., Arnander C. et al. Laser Doppler Surveillance of the Circulation of Free Flaps and Replanted Digits. Microsurgery, 6 : pp 11-19, 1985.
22. Hovius S.E.R., Adrichem L.N.A. van, Heijden P.M.A. van der et al. Postoperative Monitoring of Allogeneic Limb Transplantation in Rats. Annals of Plastic Surgery, vol. 21., no. 6: pp. 559-565, 1988.
23. Armitage P, Berry G: Factorial design. In: Armitage P. (ed) Statistical Methods in Medical Research. Blackwell Scientific Publ. London, UK: 1987: 227.
24. Skanes S.E., Samulack D.D. and Daniel R.K. Tissue Transplantation for Reconstructive Surgery. Transpl. Proc., vol. XVIII, no. 4: pp. 898-890, 1986.
25. Stevens H.P.J.D., Hovius S.E.R., Heeney J.L., et al. Immunological aspects of Partial Hand Transplantation in the Rhesus monkey. Transpl. Proc., vol. 22 (4): 2006-2008, 1990.
26. Hovius S.E.R., Stevens H.P.J.D., Peters B. and Nierop P.W.M. van. A protective thermoplast splint for the forearm and hand in non-human primates. Lab. Animals, no. 25: 1-3, 1991.
27. Hovius S.E.R., Sluimers J.E., Adrichem L.N.A. van, et al. The radial forearm flap.
    The Neth. J. of Surgery, 40-3, pp. 69-75, 1988.
28. Hovius S.E.R., Nierop P.W.M. van, Stevens H.P.J.D., et al. Allogeneic transplantation of the radial side of the hand in the rhesus monkey. II: Functional Aspects. Plast Reconstr Surg (accepted).
29. Stevens H.P.J.D., Hovius S.E.R., Nierop P.W.M. van, et al. Immunological aspects and complications of composite tissue allografting for upper extremity reconstruction: a study in the rhesus monkey. Transpl. Proc., vol. XXIII, no. 1, 1991.
30. Black K.S., Hewitt C.W., Aniel M., et al. Neuromuscular Capabilities in Long-Term Composite Tissue Allografts. Transpl. Proc., vol. XX, no. 2, suppl. 2 (April), pp 269-271, 1988.
31. Vreeswijk W., Balner H. Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation, vol. 30 (3), p. 196, 1980.
32. Cosimi A.B., Burton R.C., Colvin R. et al. Treatment of acute renal allograft rejection with OKT3 antibody. Transplantation, 32, p. 535, 1981.
33. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. The New England J. of Med., vol. 313, no. 6, pp 337-342, 1985.
34. Borleffs J.C.C., By-Aghai Z. de, Marquet R.L.. Beneficial influence of Cyclosporine A and standard immunosuppression on kidney graft survival in transfused rhesus monkeys. Transplantation, no. 32,

p. 161, 1981.
35. Hewitt C.W., Black K.S., Stenger J.A., Go C., Achauer B.M., Martin D.C. Comparison of Kidney, Composite Tissue, and Skin Allograft Survival in Rats Prolonged by Donor Blood and Concomitant Limited Cyclosporine. Transpl. Proc., vol. XX, no. 3, suppl 3 (June): 1110-1113, 1988.
36. Black K.S., Hewitt C.W., Woodard T.L. et al. Efforts to enhance survival of limb allografts by prior administration of whole blood in rats using a new survival end-point. J. of Microsurg., no. 3, pp. 162-167, 1982.
37. Hewitt C.W., Black K.S., Aguinaldo A.M.A. et al. Pathologic Alterations in the Skin Component of Composite Tissue and Skin Allografts Treated with Cyclosporine. Transpl. Proc., vol. XX, no. 3, suppl 3 (June), pp 1003-1004, 1988.
38. Samulack, D.D., Munger B.L., Dykes R.W., Daniel R.K. Neuroanatomical evidence of reinnervation in primate allografted (transplanted) skin during cyclosporine immunosuppression. Neuroscience Letters, 72: 1-6, 1986.
39. Samulack D.D., Dykes R.W., Munger B.L. Neurophysiologic Aspects of Allogeneic Skin and Upper Extremity Composite Tissue Transplantation in Primates. Transpl. Proc., vol. XX, no. 2, suppl 2 (April): 279-290, 1988.
40. Kahan B.D. et al. Complications of Sandimmun' therapy. Wld. J. Surg., 10, pp 348-360, 1986.

CHAPTER III: PARTIAL HAND TRANSPLANTATION
STUDY B : Immunological aspects

H.P.J.D. Stevens & S.E.R. Hovius

B.1 Introduction

Since Cyclosporine A (CyA) has been introduced for experimental organ grafting in 1977[1] and for clinical use in 1978[2], it has been used successfully for the suppression of the host immune response after allogeneic transplantation of many organs, experimentally[3] as well as clinically[4-7]. In the last two decades, as a consequence of the recent advances that have been made in clinical microsurgery, it also became feasible to explore the possibility of surgical reconstruction of the upper extremity with composite tissue allografts (CTAs)[8-12,subm]. Allogeneic limb transplantation in the rat has been performed successfully with long term allograft survival, provided that treatment with moderate doses of CyA was continuous[8,9]. Tolerance to limb allografts in rats could, probably, partly or totally be attributed to the development of donor-host lymphoid chimeras[10]. However, for the study into the feasibility of allogeneic hand transplantation in man, research in rodents only yields limited information and results can not be extrapolated directly to the situation man. For anatomical, functional, immunological and phylogenetic reasons the nonhuman primate is obviously a more representative experimental animal.

So far only two hand transplantation studies in the nonhuman primate (baboon) have been reported[11,12]. In this species, even high doses of CyA in combination with Prednisolone (DAF) could not prevent rejection of the allogeneic hand transplant in the majority of the cases, nor could a rise in steroid treatment reverse all rejection episodes adequately. Therefore, in the allogeneic partial hand transplantation model in the rhesus monkey, a more specific anti-rejection therapy was incorporated, apart from continuous administration of CyA and DAF. A combination of monoclonal antibodies (MAbs) was designated with high immunosuppressive potential, consisting of seven MAbs specific for CD3, CD4, CD8 and major histocompatibility complex (MHC) class II antigens. It was demonstrated previously, that this combination of MAbs prolonged skin allograft survival times significantly from 8.3 (SD=0.7) to 19.3 (SD=1.8) days[13]. MAbs are relatively new and very selective tools in modulating the immune response. Especially anti-leucocyte MAbs have proven to be effective in reversing organ allograft rejection in the non-human primate[14,15] as well as in man[15,16]. Since preoperative third party blood transfusions are known to prolong allograft survival[17,18] even under CyA treatment[19], the value of such blood transfusions was tested also. This study focussed on the general, intravascular and intra-transplant effects of modulating the host immune response against a CTA by the afore mentioned agents.

B.2 Material and methods

B.2.1 Animals

Adult rhesus monkeys (Macaca mulatta), ranging from 7 to 21 years in age and not alloimmunized prior to these experiments, were used. Unrelated donor-recipient combinations were mismatched for rhesus MHC class I and II antigens.

B.2.2 Experimental model

The transplant consisted of the first ray of the hand (the thumb) enlarged with a radial forearm flap of approximately 20 cm^2 (submitted[4]). Dissection and amputation of the partial hands were performed under sterile conditions, using tourniquet hemostasis and an antibiotic prophylaxis. All donor structures (metacarpal bone, radial artery, cephalic vein, tendons, nerves and skin) were reconnected at the appropriate recipient sites. Postoperatively, the extremities were immobilized in a custom made arm protective thermoplast splint[20].

Table 1. The composition of the combination of monoclonal antibodies (MAbs) that was used for the treatment of rejection episodes of allogeneic partial hand transplants in the rhesus monkey[a].

MAb	specificity[b]	dose (mg/kg)	reference
B8.12	MHC-DR	1	22
7.5.10.1	MHC-DR,-DQ,-DP	1	23
5B11	CD3	0.5	24
FN18	CD3	0.5	22
RIV6	CD4	0.5	25
RIV7	CD4	0.5	25
FK18	CD8	0.5	26

[a]For treatment of rejection episodes this combination of seven MAbs was administered once daily as an intravenous bolus injection for a period of ten days.

[b]Abbreviations used: MHC = Major Histocompatibility Complex antigens class II; CD = Cluster of Differentiation as defined during the Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989).

B.2.3 Experimental groups and immunosuppression

This study concerned 12 successful transplantations of the partial hand. Recipients were divided randomly into four different treatment subgroups following a statistical design according to Fisher[21] in order to economize on monkeys and enable evaluation of the effects of preoperative third party blood transfusions and MAb anti-rejection therapy (Table 2). Basic immunosuppressive treatment starting one day preoperatively consisted of (s.c.) administration 25 mg/kg/day CyA in Miglyol-812. An initial high dose of 12 mg/kg/day of DAF was added (im), starting on the day of operation and tapered every three days until a maintenance dose of one mg/kg/day was reached at the 12th postoperative day.

If rejection was observed macroscopically (swelling, erythema and blister formation) and could be confirmed histologically, in subgroups I and II, rejection was treated with a combination of MAbs, administered once daily as an intravenous bolus injection for a period of ten days. Its composition is presented in Table 1 together with the relevant specificity of each MAb and the concentration used for injection. All MAbs used in this study are crossreactive with rhesus lymphocytes[22,24,25,subm].

In subgroups III & IV, rejection was treated by increasing the DAF dose to 12 mg/kg/day again. Tapering of the DAF dose was done as described above.

The therapeutic value of these anti-rejection treatment protocols was tested, as well as the value of preoperative third party blood transfusions (Table 2). Blood transfusions consisted of 20 ml fresh citrated whole blood from donors, fully mismatched for rhesus MHC class I and II antigens of both graft donor and recipient. Three third party blood transfusions were given at biweekly intervals (-6,-4,-2 weeks) before transplantation to monkeys in treatment subgroups I & III. Endpoint of experiment was reached when either the allograft was completely rejected or the graft recipient died.

B.2.4 Assessment of peripheral blood parameters

By means of a specific radioimmuno-assay (RIA, Sandimmun-Kit; Sandoz Ltd., Basle, Switzerland)[27] it was demonstrated that whole blood CyA trough levels were above the minimal required concentration of 400 ng/ml[28] in 83% of all postoperative samples and in 92% of all samples after day five. (Correction factor to compare our data measured by the monoclonal RIA with data measured by the polyclonal RIA is set to 50%[29]). CyA levels from day 5 to start of anti-rejection therapy did not differ significantly between long term and short term survivors (P > 0.05, Mann-Whitney U test). Median CyA levels were 861 (SD =

272) and 676 (SD=256) ng/ml, respectively.

Levels of circulating injected MAbs and titers of antibody formation against the injected MAbs were determined using a sandwich enzyme-linked immunosorbent assay technique (ELISA) as described previously[30].

All allograft recipients were monitored frequently with respect to total leucocyte and lymphocyte counts and relative numbers of CD3+, CD4+, CD8+ and MHC class II+ cells, using an indirect immunofluorescence technique and analysis by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA) as described elsewhere[31].

B.2.5 Histological studies

After operation skin biopsies were taken from the transplant weekly. During rejection, biopsies were taken two to three times per week. After excision with a 3 mm^2 biopsy stance, one half of the biopsy was fixed in buffered formalin and processed routinely on hematoxylin-azofloxine stained sections. Severeness of rejection was scored semiquantitatively; light (+) for slight edema, small infiltrates of lymphocytes and histiocytes; moderate (++) for diffuse edema, larger lymphohistiocytic infiltrates, capillary destruction, hemorraghes, (sub)epidermal bulla formation; severe (+++) for massive lymphohistiocytic infiltrates, extensive hemmorraghe, epidermiolysis; complete graft rejection (CGR) was typified by a severe rejection score plus crust formation.

The remaining other half of the biopsy, snapfrozen in liquid nitrogen chilled isopentane, was used for parallel immunohistochemical studies. Technique and a semiquantitative scoring method for distribution and intensity of staining are described previously[30,subm]. A previously described selection of MAbs[30] was used to demonstrate expression of the following rhesus antigens: MHC class I, MHC class II-DR, CD2 (pan T cell), CD4 and CD8. In control incubations the primary antibody was omitted.

B.2.6 Statistical analysis.

When appropriate, Fisher's exact test, Mann-Whitney U test, two sample t-test, and log-rank test were performed for hypothesis testing. Differences were considered significant if $P < 0.05$.

B.3 Results

B.3.1 Allograft survival times and rejection

Twelve allogeneic transplantations of the partial hand were performed successfully. Graft survival times were short in six cases (21-33 days) and long in another six (79-179 days; Table 2, Fig. 1a).

Third party blood transfusions did not induce a significant difference in the moment of onset of allograft rejection (days of onset of rejection in groups I and III versus those in groups II and IV: P > 0.05, t-test).

Table 2. Allogeneic partial hand transplantation in 12 rhesus monkeys treated with CyA, steroids, third party blood transfusions and monoclonal antibodies.

Protocol[a]	Monkey	Start of rejection therapy (days)	Rejection reversed	Graft survival time (days)
I:	2799	79	-	79[c]
MAbs and	4023	62	+	
bloodtrf.		111[b]	+	121[c]
	2988	15	-	22[c]
II:	3992	139	-	144
MAbs,	3439	41	+	
no bloodtrf.		75[b]	+,partially	97[c]
	3308	-		179[c]
III:	2596	13	-	30
DAF and	2I	21	-	29[c]
bloodtrf.	3310	-		85[c]
IV:	1FU	21	-	33
DAF,	1550	21	-	33
no bloodtrf.	3212	12	-	21

[a]**Immunosuppressive protocol, abbreviations used: MAbs:** anti-rejection therapy consisted of a combination of seven monoclonal antibodies, as described in Table 1; **bloodtrf.:** three third party blood transfusions were given to the recipient, preoperatively; **DAF:** anti-rejection therapy consisted of a raise in steroid treatment.
[b]second rejection episode in monkeys #4023 and #3439, respectively.
[c]death of rhesus monkey (spontaneously or due to euthanasia).

Blood transfusions neither facilitated reversal of graft rejection (P > 0.05, Fisher's exact test) nor had a significant effect on allograft survival, in this study (P > 0.05, log-rank).

Overall, rejection of the allograft occurred in ten partial hand recipients. Five monkeys received the combination of MAbs for treatment of rejection crises. In two out of these five monkeys, graft rejection could be reversed completely (Fig. 1b-d).

Also a second rejection episode was reversible, although in one monkey only partially, using the same combination of MAbs. In two out of the three monkeys, where the MAbs therapy had not reversed graft rejection, MAbs had not been administered for the proposed duration of ten days. Due to side effects like drowsiness and vomiting, one monkey received four doses on alternating days, the other monkey, unfortunately, died due to a shock after the first dose of MAbs. In five other recipients, graft rejection was treated by increasing the DAF dose. In none of these cases, graft rejection could be reversed. There was no significant difference in the moment of onset of allograft rejection in groups I & II compared to groups III & IV (P > 0.05, log-rank test). However, if MAb anti-rejection therapy was used (groups I & II), graft survival was prolonged significantly longer than if rejection was treated by a raise in steroids (groups III & IV, P = 0.015, log-rank test).

B.3.2 Effects on peripheral blood parameters: Antibody serum levels

Serum trough levels of the injected MAbs varied from four to 120 ng/ml during the period of MAbs administration. Moreover, in all monkeys serum levels remained detectable up to three to ten days after the last injection. In both monkeys where the episodes of allograft rejection could be reversed and CyA and DAF treatment was continued, no neutralizing monkey-anti-mouse antibodies could be detected.

B.3.3 Hematology and lymphocyte subsets

If rejection was treated with a raise in steroids, no leucopenia nor lymphopenia in absolute numbers was seen. However, if rejection was treated with the combination of MAbs, in all monkeys the absolute number of leucocytes was reduced to 50%, that of lymphocytes even to 14% of pretreatment levels, already within ten minutes after the first injection. Lymphopenia and leucopenia sustained till approximately six days after the first injection.
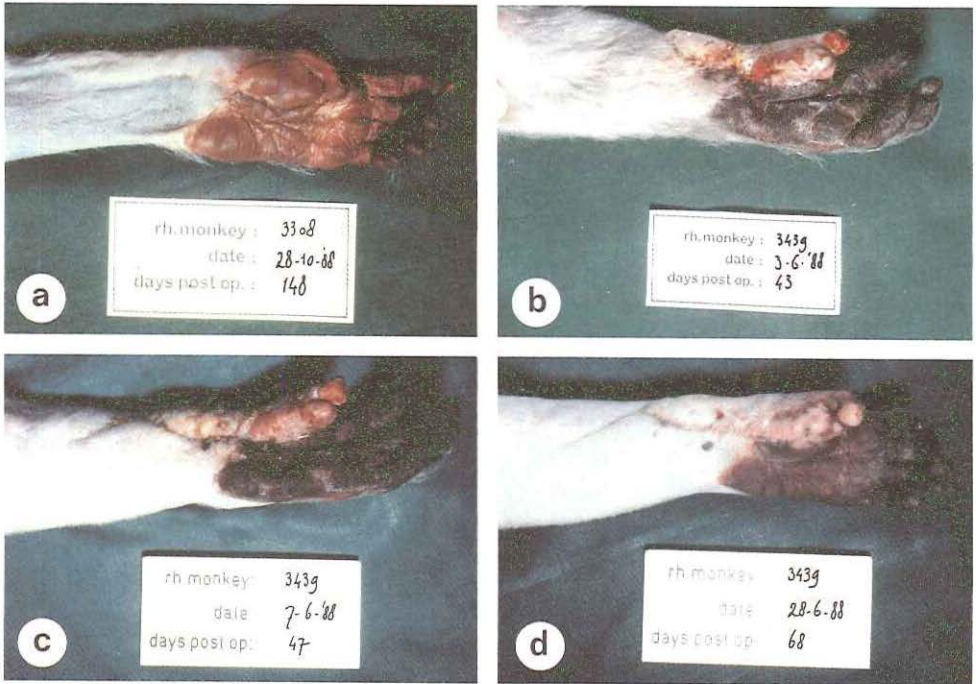
Figure 1a. Complication free graft survival of an allogeneic partial hand in monkey #3308, 148 days postoperatively.

Figure 1b. Full blown rejection with macroscopical swelling (due to edema), erythema (due to dermal hemorraghes) and blister formation (due to epidermolysis) of the allogeneic partial hand in rhesus monkey #3439, 43 days postoperatively. The 10 day course of anti-rejection therapy with the combination of monoclonal antibodies as described in Table 1, was started on day 42.

Figure 1c. 6 Days after onset of MAbs anti-rejection therapy in monkey #3439, first macroscopical signs of successful reversal of rejection are visible.

Figure 1d. 26 Days after onset of successful MAb anti-rejection therapy. The integrity of the transplant of rhesus monkey #3439 has fully been restored.

In all cases, ten minutes after the first injection of MAbs, over 90% of the peripheral blood lymphocytes (PBLs) that had not been eliminated, was coated as was demonstrated by incubating with the fluorescent goat-anti-mouse IgG alone (Fig. 2). As soon as coating of PBLs decreased, the relative number of MHC class II positive cells increased to pretreatment levels. Coating of PBLs disappeared, after approximately 20 days.
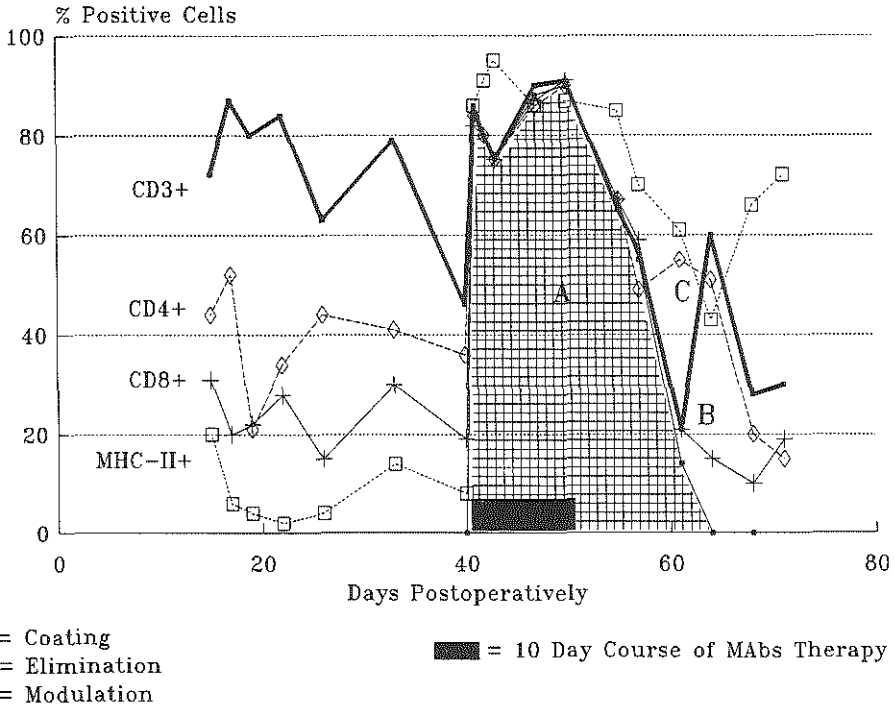
Figure 2. Lymphocyte subset fluctuations in monkey #3439, representative for the intravascular events that could be observed every time a monkey was treated with a ten day course of the combination of seven MAbs described in Table 1. MAb administration is indicated by the black bar. The surface of the shaded area 'A' depicts the relative number of coated cells. Until approximately 20 days after the first injection, no cells expressing the CD3 and CD8 surface marker are detectable (see point 'B'). Detection of CD4+ cells without the concomittant expression of CD3 (see point 'C'), indicates modulation of the CD3 surface antigen.

Elimination of CD3+ and CD8+ lymphocytes lasted approximately equally long. Modulation of the CD3 antigen occurred also (Fig 2). In the monkeys in which MAbs therapy did not reverse rejection PBLs had been eliminated to the same extent, but CD3+ and CD8+ subset-elimination lasted shorter. Also coating of PBLs was of less intensity and of shorter duration.

B.3.4 General histology

In all cases where rejection was not reversible, the allograft was completely rejected within five to ten days after onset of rejection therapy (Table 3). If reversal of rejection could be established with MAb therapy, diffuse histiolymphocytic infiltrates and dermal hemorraghes were eliminated within approximately three days. A slight focal
infiltrate of mainly lymphocytes remained (Table 3, Fig. 3c,d). When (focal) epidermolysis had occurred, complete re-epithelization developed within five days. Only after the second rejection episode, parts of the skin of the thumb of monkey #3439 became necrotic. The thenar muscle was still vital and skin defects healed within the next 14 days.

Table 3. Histological observations during anti-rejection treatment with high-dose steroids or a combination of monoclonal antibodies in rhesus monkey allogeneic partial hand recipients[a].

Anti-rejection therapy	Monkey	day -2/0[b]	day 1/4	day 5/9	day 10/15	Reversal of rejection
Steroids	2596	D++	D+++	D+++,C.G.R.		-
	2I	D+/++	D++	F++/+++[d]		N.A.[d]
	1FU	D+/++	D+/++	D+++	C.G.R.	-
	1550	F++/+++	D+++	D+++	C.G.R.	-
	3212	D++/+++	D+++	D+++	C.G.R.	-
MAbs	2799[c]	D++/++++[d]				N.A.[d]
	2988	D+/-	D+	D+++,C.G.R.		-
	3992[c]	D+/++	D++	D+++,C.G.R.		-
	4023[1st episode]	D+/++	D+/++	F+	F+/++	+
	3439[1st episode]	D++/+++	D++/+++	(n)	F+	+
	4023[2nd episode]	F-D+/++	F+/++	F-D+	F-D+	+
	3439[2nd episode]	D+	F++	F+	F+/++	+[e]

[a]Severeness of rejection was scored semiquantitatively (Materials & methods); F=focal, D=diffuse, n=normal, C.G.R.=complete graft rejection.
[b]Day 0 = day of start of anti-rejection therapy
[c]Monkeys #2799 and #3992 received one and four doses of MAb therapy, respectively
[d]Due to death of the monkey reversal of graft rejection was not assessable (N.A.)
[e]This time reversal of rejection was partial: a part of the volar side of the thumb was rejected

B.3.5 Immunohistology

During anti-rejection treatment with a raise in steroids, changes in relative numbers of CD2+, CD4+ and CD8+ lymphocytes did not correlate significantly with parameters associated with clinical rejection. Succesful MAb anti-rejection therapy caused coating of graft infiltrated lymphocytes, histiocytes as well as dermal and epidermal cells (Fig. 3a,b). This coating lasted from the first to approximately the sixth day after onset of MAb therapy. Moreover, successful MAbs therapy eliminated the majority of the rejection infiltrate which was predominantly composed of CD2+CD8+ lymphocytes.

Until the next rejection episode, numbers of infiltrated cells remained low, mainly located perivascularly (Fig. 3c,d). In those monkeys where the MAb therapy could not reverse allograft rejection, coating and elimination all lasted shorter. Altough lymphocytes were first eliminated vigorously, they returned in pretreatment ratios and numbers within four to six days, whereas if anti-rejection therapy was successful this would last ten to 28 days. It was further noted that HLA class I and II-DR antigen expression was increased during rejection on all cells in the allograft. If rejection could be reversed, MHC class II-DR expression was reduced vigourously while MHC class I antigen expression was also reduced but to a lesser degree.

B.3.6 Complications of the immunosuppressive therapy

Major side effects of the immunosuppressive therapy concerned the death of seven monkeys of which one died due to shock, three died due to lymphoid tumor development and three due to opportunistic bacterial infections. Further analysis of what factors might have influenced this high rate of death are presented elsewhere[32].

B.4 Discussion

Technically, transplantation of the (partial) hand in the nonhuman primate can be realized and functional recovery can be obtained[11,12,subm]. However, before such an operation can be performed in the human being, many problems should be solved first. Most importantly, long term uneventful graft survival can still not be realized. This study focussed on the general, intravascular and intra-transplant effects of some of the most potent immunosuppressants available today in a partial hand transplantation model in the rhesus monkey.
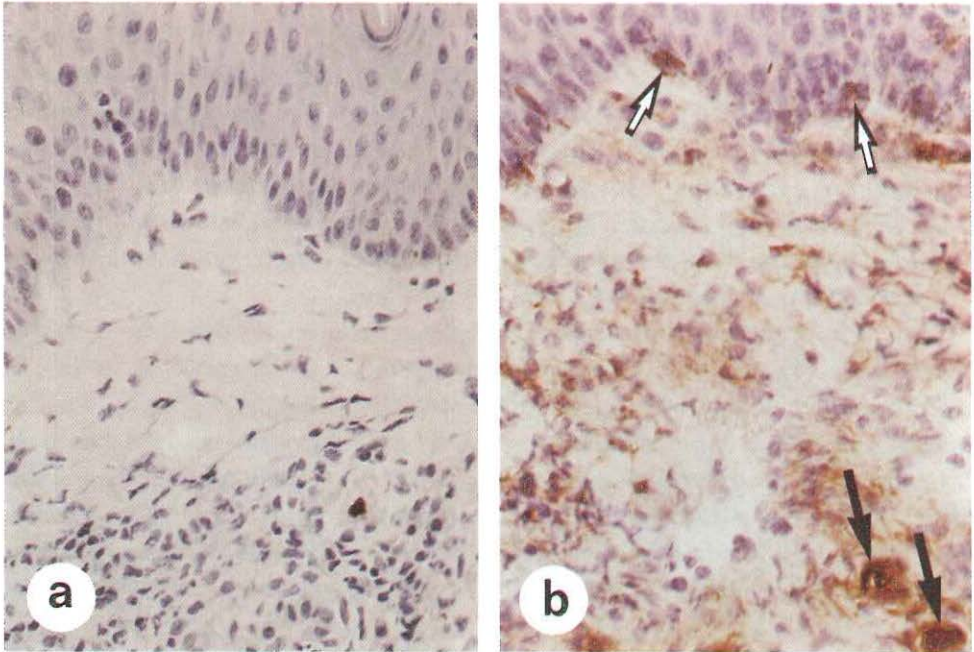
Figure 3a. Negative control section for immunohistology from the transplant of monkey #4023, 61 days postoperatively, during standard CyA and DAF therapy. Primary antibody was omitted in the staining procedure. Neither cellular infiltrates nor pre-existing tissue is positively stained
Figure 3b. Negative control section for immunohistology from the transplant of monkey #4023, 65 days postoperatively, three days after onset of MAbs anti-rejection therapy. Intravascularly, agglutination of coated lymphocytes is visible (closed arrows). Also, extravacular lymphocytes are coated by the injected antibodies. Even, cells in the epidermis are coated (open arrows).

Various recent studies in nonhuman primates stressed the inevitability to use continuous high doses of CyA (20-48 mg/kg/day) to obtain prolonged allograft survival of CTAs[11,12,28]. In the present study, 25 mg/kg/day CyA in combination with a maintenance dose of steroids was used, yielding allograft survival times ranging between 21 and 179 days. Based on survival times of fully mismatched skin grafts in the rhesus monkey, it can be assumed that without this base line immunosuppression allografts would have been rejected within 7 to 10 days[13,33].
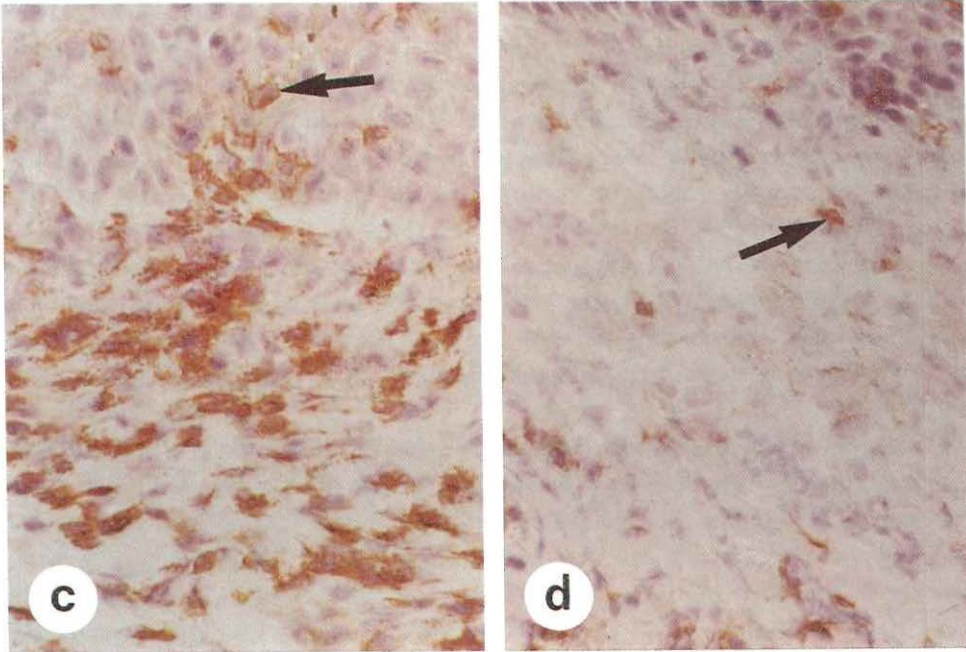
Figure 3c. A biopsy from the transplant of monkey #3439, 71 days after operation at the time of onset of rejection, histochemically stained with anti-CD8 MAb. The majority of the rejection infiltrate is composed of CD8+ T-cells. Also lymphocytes infiltrated into the epidermis are of the CD8 phenotype (arrow).

Figure 3d. A transplant biospy from the same monkey, 11 days later, seven days after onset of MAb anti-rejection therapy and stained with the same MAb, shows that the majority of the rejection infiltrate has been eliminated. Only perivascularly, a few CD8+ lymphocytes are still present (arrow, in all cases magnification: 300x).

The absence of a protective effect of third party blood transfusions on CTA survival, could be due to the usage of high doses of CyA and DAF masking the expected effect. Alternatively, the difference in antigenicity of a CTA including skin compared to a kidney or heart allograft might have impaired an anticipated transfusion effect[34].

Anti-rejection therapy with a combination of MAbs, specific for CD3+, CD4+, CD8+ and MHC class II-positive cells, was demonstrated to prolong CTA survival significantly longer than conventional anti-rejection therapy by a raise in steroids. These results confirm other clinical kidney and liver transplantation studies in which the MAb OKT3 (CD3-specific) was more effective in reversing graft rejection than anti-rejection therapy with steroids[35]. Furthermore, also in two monkeys a second rejection episode could be reversed successfully, using the same combination of MAbs. Most likely this was achievable due to the fact that, in this study, no neutralizing monkey-anti-mouse antibodies were formed in any of the monkeys in which CyA and DAF therapy was continued during and after reversal of allograft rejection. Without CyA and DAF, the same MAbs used in a skin transplantation study, did induce an antibody response detectable from the 7th day after the first injection onwards[13].

Baseline therapy with CyA and DAF nor anti-rejection therapy with steroids induced significant fluctuations in the relative and absolute number of leucocytes and PBLs. MAbs anti-rejection therapy, however, did induce leucopenia as well as lymphopenia in a similar degree as was found in previous skin transplantation studies[13,37]. Most likely, due to the absence of an antibody response, elimination of T cells and coating of all remaining cells lasted 10 to 20 days longer and subsequent modulation of the CD3 antigens occurred later than in previous studies[13,37].

Coating of cells within the allograft lasted for three to six days which is about 14 to 16 days shorter than the observed coating of PBLs. At this time, serum levels of injected MAbs fluctuated between four and 45 ng/ml. Apparently, once the majority of the rejection infiltrate was eliminated within approximately three days, diffusion of the circulating MAbs into the allograft became more difficult. This could have been due to a decrease in vascular permeability. This would correlate well with the observation that edema minimalized simultaneously.

Elimination of the CD2+CD8+ lymphocyte subset from the allograft as well as from the peripheral blood, correlated best with reversal of rejection and thus with prolongation of allograft survival times. It therefore, seems likely that CD8+ T cells play a major role in the process leading to graft destruction of a fully mismatched CTA in the rhesus monkey. Nevertheless, anti-CD8 as well as anti-CD4 MAbs are both known to prolong skin allograft survival[13,37] implying involvement of both CD4+ and CD8+ cells in the procesess rejection. Possibly, it is a matter of preference of the host to reject fully mismatched CTAs by a cell mediated cytotoxicity dominated by CD8+ cells, despite alternatives[38].

When MAbs therapy reversed rejection, MHC antigen expression in the allograft was reduced or even completely disappeared. The elimination of the rejection infiltrate, that

tended to occur slightly ahead MHC antigen decrease, may have abrogated local lymphokine production, hindering the upregulation of MHC antigen expression and associated allogeneicity of the graft. These findings are in line with previous data on MHC antigen modulation during acute rejection of skin[30] and liver allografts[14].

As seven monkeys died during experiment, one might assume that the immunosuppressive treatment was too aggressive[32]. However, addition of MAbs anti-rejection therapy to baseline immunosuppression did not correlate to incidence of death, significantly[32]. Similar doses of CyA and DAF were administered for periods over one year in baboons and no such side effects were reported[11,12]. Momentarily, it is under investigation whether inflicted case histories of the monkeys involved, might have precipitated the (earlier?) occurrence of the side effects.

In conclusion, technical feasibility and the fact that recovery of function can be achieved, bring transplantation of the hand for reconstruction of upper extremity deformities, closer to reality. But at the same time, even continuous high doses of the most potent immunosuppressiva available today can not guarantee uneventful graft survival. Promisingly, MAb therapy could reverse rejection and prolonged allograft survival times significantly in fully mismatched donor/recipient combinations in the rhesus monkey. Graft reversal was associated with a dramatic elimination of CD2+,CD3+ and CD8+ lymphocytes in the peripheral blood as well as in the allograft itself. However, before actual transplantation of the hand can be performed in the human being, to our opinion a more adequate, less toxic immunosuppressive regimen should become available first.

B.5 References

1. Calne RY, Rolles K, White DJG. Cyclosporine A - a powerful immunosuppressant in dogs with renal allografts. IRCS Med Sci Surg Transplant 1977; 5: 595.
2. Calne RY, White DJG, Thiru S, et al. Cyclosporin A in patients receiving renal allografts from cadaver donors. Lancet 1978; 2: 1323-1327.
3. Green CJ. Experimental transplantation and cyclosporine. Transplantation 1988; 46: 3S.
4. Merion RM, White DJG, Thiru S, et al. Cyclosporine: Five years' experinece in cadaveric renal transplantation. N Eng J Med 1984; 310: 148.
5. McGregor CGA, Oyer PE, Shumway NE. Heart and heart-lung transplantation. Prog Allergy 1986; 38: 346.
6. Starzl TE, Iwatsuki S, Shaw BW Jr, et al. Orthotopic liver transplantation in 1984. Transplant Proc 1985; 17: 250.
7. Ringden O. Cyclosporine in allogeneic bone marrow transplantation. Transplantation 1986; 42: 445.
8. Fritz WD, Swartz WM, Rose S, Futrell JW, Klein E. Limb allografts in rats immunosuppressed with cyclosporine A. Ann Surg 1984; 199(2): 211.
9. Black KS, Hewitt CW, Fraser LA, et al. Composite tissue (limb) allografts in rats. II. Indefinite

survival using low-dose Cyclosporine. Transplantation 1985; 39(4): 365.

10. Hewitt CW, Black KS, Dowdy SF, et al. Composite tissue (limb) allografts in rats. III. Development of donor-host lymphoid chimeras in long-term survivors. Transplantation 1986; 41(1): 39.

11. Daniel RK, Egerszegi EP, Samulack DD, Skanes SE, Dykes RW, Rennie WRJ. Tissue transplants in primates for upper extremity reconstruction: a preliminary report. J Hand Surg 1986; 11A(1):1.

12. Stark GB, Swartz WM, Narayanan K, Moller AR. Hand transplantation in baboons. Transplant Proc 1987; 19(5): 3968.

13. Stevens HPJD, Roche N, Hovius SER, Jonker M. In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive cells. Transpl Proc 1990, 22(4): 1783.

14. Steinhoff G, Jonker M, Gubernatis G, et al. The course of untreated acute rejection and effect of repeated anti-CD3 monoclonal antibody treatment in rhesus monkey liver transplantation. Transplantation 1990; 49(4): 669.

15. Cosimi Ab. Anti-T-cell monoclonal antibodies in transplantation therapy. Transpl Proc 1983; 15(3): 1889.

16. Klein JB, McLeish KR, Bunke CM, et al. Use of OKT3 monoclonal antibody in the treatment of acute cardiac allograft rejection. Transplantation 1988; 45: 727.

17. Borleffs JCC, Marquet RL, By-Aghai Z de, Vreeswijk W van, Neuhaus P, Balner H. Kidney transplantation in rhesus monkeys: matching for D/DR antigens, pretransplant blood transfusions and immunological monitoring before transplantation. Transplantation 1982; 33: 285.

18. Opelz G, Terasaki PI. Importance of preoperative (not peroperative) transfusions for cadaver kidney transplants. Transplantation 1981; 31: 106.

19. Hunsicker LG, Schanbacher BA, Goeken NE, Corry RJ. A randomized comparison of preoperative and intraoperative transfusions in recipients of first cadaveric renal allografts. Transpl Proc 1988; XX (1, S1): 286.

20. Hovius SER, Stevens HPJD, Peters B, Nierop PWM van. A protective thermoplast splint for the forearm and hand in nonhuman primates. Lab Animals 1990, 24: 380.

21. Armitage P, Berry G. Factorial design. In: Armitage P eds. Statistical methods in medical research. London, UK: Blackwell Scientific Publications, 1987: 227.

22. Nooij FJM, Borst JG, Meurs GJE van, Jonker M and Balner H. Differentiation antigens on rhesus monkey lymphocytes. I. Identification of T cells bearing CD3 and CD8, and of a subset of CD8 bearing cells. Eur J Immunol 1986; 16: 975.

23. Koning F, Schreuder GMTh, Bontrop R, et al. A monoclonal antibody with MT2 specificity. Disease Markers 1984; 2: 75.

24. Jonker M, Slingerland W. Reactivity of monoclonal antibodies specific for human CD markers with rhesus monkey leucocytes. In: Knapp W, Dorken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, vd Borne AEGKr, eds. Leucocyte Typing IV. Oxford University Press; 1990: 1058.

25. Jonker M, Nooij FJM, Chatenoud L. Immunosuppression by monoclonal T cell antibodies. Transpl Proc 1986; 18(5): 1287.

26. Koning F, Fifth European Immunology meeting, 1982

27. Holt DW, Marsden JT, Johnston A. Measurement of cyclosporine: methodological problems. Transpl Proc 1986; 18(S5): 101.

28. Skanes SE, Samulack DD and Daniel RK. Tissue transplantation for reconstructive surgery. Transpl Proc 1986; 18(4): 898.

29. Holt DW, Johnston A, Marsden JT. Monoclonal antibodies for radioimmunoassay of Cyclosporine: a multicenter comparison their performance with the Sandoz polyclonal radioimmunoassay kit. Clin Chem 1988; 34(6): 1091.

30. Stevens HPJD, Kwast ThH van der, Meide PH van der, Vuzevski VD, Buurman WA, Jonker M. Synergistic immunosuppressive effects of monoclonal antibodies specific for interferon gamma and tumor necrosis factor alpha. A skin transplantation study in the rhesus monkey. Transplantation 1991; (in press).
31. Jonker M, Goldstein G, Balner H. Effects of in vivo administration of monoclonal antibodies specific for human T cell subpopulations on the immune system in a rhesus monkey model. Transplantation 1983; 35(6): 521.
32. Stevens HPJD, Hovius SER, van Nierop PWM, Jonker M, Heeney JL. Immunological aspects and complications of composite tissue allografting for upper extremity reconstruction: a study in the rhesus monkey. Transpl Proc (in press)
33. Vreeswijk W, Balner H. Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 1980; 30 (3): 196.
34. Hewitt CW, Black KS, Stenger JA, Go C, Achauer BM, Martin DC. Comparison of kidney, composite tissue and skin allograft survival in rats prolonged by donor blood and concomitant limited cyclosporine. Transpl Proc 1988; 20 (3,S3): 1110.
35. Starzl TE, Fung JJ. ORTHOCLONE OKT3 in treatment of allografts rejected under Cyclosporine-steroid therapy. Transpl Proc 1986; 18: 937.
36. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. New Eng J Med 1985; 313 (6): 337.
37. Nooij FJM, Jonker M. The effect on skin allograft survival of a monoclonal antibody specific for a polymorphic CD3-like cell surface molecule in rhesus monkeys. Eur J Immunol 1987; 17: 1089.
38. Auchincloss H Jr, Mayer Th, Ghobrial R, Winn HJ. T-cell subsets, bm mutants and the mechanisms of allogeneic skin graft rejection. Immunol Res 1989; 8: 149.

CHAPTER III: PARTIAL HAND TRANSPLANTATION
STUDY C   : Functional aspects

S.E.R. Hovius & H.P.J.D. Stevens

C.1 Introduction

Surgeons are often confronted by their inability to replant a severed crushed hand. Allogeneic hand transplantation could provide a solution for such a case. This procedure would, however, only be indicated, if uneventful long term graft survival and functional recovery could be achieved. Experimental studies of composite tissue allografts should therefore be performed in which functional recovery could be tested thoroughly.
Following limb transplantation in rats, sciatic nerve stimulation proximal and distal to the nerve repair showed contraction of the soleus and gastrocnemius muscles[1,2]. More recently, sensory and motor recovery has been demonstrated in allogeneic hand transplants in baboons[3, 4]. Electrophysiological tests in those studies revealed sensory reinnervation of transplanted skin as well as reinnervation of donor muscle following electrical stimulation of the recipients median and ulnar nerve[5, 6]. However their animals were not conscious during sensory reinnervation testing and there was no longitudinal evaluation of functional recovery.

Technical feasibility of allogeneic transplantation of the radial side of the hand in the rhesus monkey has been previously demonstrated[7]. In this study, data are presented regarding longitudinal studies of sensory and motor reinnervation of the composite tissue allograft.

C.2   Material and Methods
C.2.1 Animals

Rhesus monkeys (Macaca Mulatta) were born and raised at the TNO Primate Center, Rijswijk, the Netherlands, employing a "harem-type" breeding system. Average weight was 7.5 kg, age varied from nine to 24 years and male to female ratio was two to one. The animals were selected on absence of prior alloimmunization, on normal kidney and liver function and non-injured, relatively large hands. If possible left or right handedness was assessed.

Animal care complied with the "Principles of Laboratory Animal Care" and the "Guide for the care and use of laboratory animals" (NIH publication no. 80-23, revised 1978).
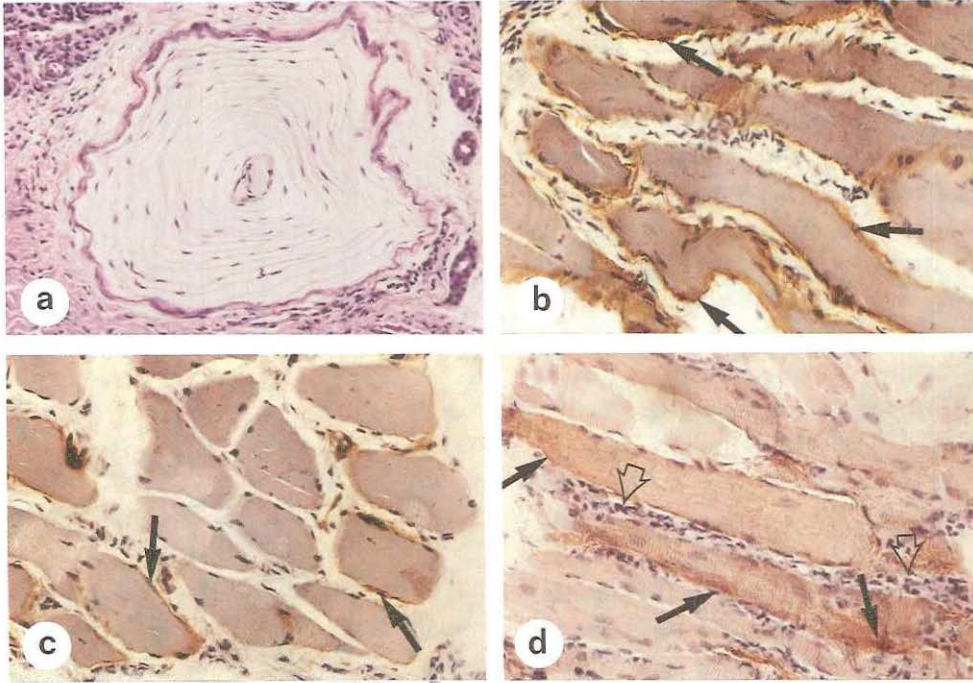
Figure 5a. H&A stained section of the thenar of the allograft of monkey 4023, 114 days postoperatively, three days after the onset of the second anti-rejection therapy. Vital foreign Pacinian corpuscles are still present (magnification 300x). **Figure 5b.** Enzymhistochemical staining for acetylcholine-esterase activity demonstrating the distribution of vital motor end plates over the muscle of monkey BM in the unoperated thenar muscle. The black lining on the surface of the muscle cells (see arrows) indicates the positively stained motor end plates (magnification 300x). **Figure 5c.** With the same technique as in 5b, the thenar muscle of monkey BM, 18 months after autologous replantation shows a comparable staining pattern on the majority of its muscle cells (arrows, magnification 300x). **Figure 5d.** With the same technique as in 5b, the thenar muscle of monkey 3310, 85 days after allogeneic transplantation also clearly shows positive motor end plates (closed arrows). Some rejection infiltrate is present (open arrow; magnification 300x).

C.2.2 Experimental model

The transplant consisted of the first ray of the hand (the thumb) enlarged with a radial forearm flap and thus involved bone, muscles, vessels, nerves and skin.

Following amputation at the donor site all structures were reanastomozed to corresponding structures at the recipient site of another animal (figure 1a). Detailed information about the transplantation technique has been previously described[7]. Recovery of function of the transplant was evaluated in three adult monkeys following autologous replantation and in twelve adult rhesus monkeys following allogeneic transplantation. Autologous replantations had been carried out to demonstrate the feasibility of the research model (submitted). The functional recovery of these three monkeys was also used as a control for the twelve transplantation cases.

For evaluation of sensory recovery, only the median nerve distribution area was considered, as it showed the most consistent anatomy at the level of the junction of the sensory and motor branch to the thumb. A nerve anastomosis in this area proved to be very reliable, enabling good comparison of reinnervation between the different monkeys. The proximal part of the radial forearm flap is mainly innervated by the lateral antebrachial cutaneous nerve. This nerve was not reanastomozed, the corresponding skin area served as a control. For evaluation of motor recovery the thenar muscle action was investigated. Although handedness in macaques is not as clear as in chimpanzees and man[8, 9, 10], the dominant hand was determined preoperatively by assessing which hand was mostly used by
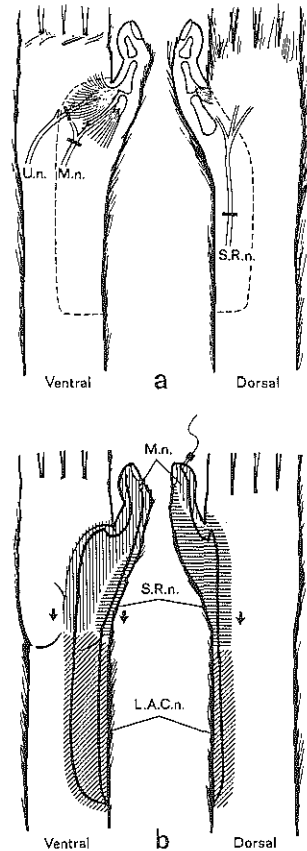
Figure 1a. Research model. The dotted line marks the radial side of the hand with the radial forearm flap. Nerves: U.n. = ulnar nerve; M.n. = median nerve; S.R.n. = superficial radial nerve.

Figure 1b. Sensory recovery was assessed by means of a small electric current applied locally to the skin of the conscious monkey to provoke a withdrawal reflex. The measuring sites were 5 mm apart along the indicated thick black lines on the model. The skin areas corresponding to the appropriate nerves are marked. M.n. = median nerve; S.R.n. = superficial radial nerve; L.a.c. = lateral antebrachial cutaneous nerve.

the monkey to retrieve food offered from different directions. To stimulate functional recovery, the hand of preference was operated upon.

The experiment was terminated when there was complete rejection of the transplant or death of the monkey.

C.2.3 Immunosuppression

Basic immunosuppression consisted of continuous high doses of Cyclosporin A (CyA, 25 mg/kg/d) in combination with an initial high dosage of prednisone (DiAdreson Faquosum (DAF), 12 mg/kg/d), which was decreased stepwise to a maintenance dosage of 1 mg/kg/d in twelve days. Swelling, erythema and blister formation were considered to be macroscopical signs of rejection. After histological confirmation (oedema, epidermolysis, dermal hemorrhage and lymphohistiocytic infiltrate) anti-rejection therapy was started. In half of the animals, anti-rejection therapy consisted of a 10 day course of a combination of monoclonal antibodies specific for different immunocompetent cells[11, 12]. In the other half, rejection was treated by increasing the DAF dosage to 12 mg/kg/d, again followed by a decrease to maintenance doses as described above.

C.2.4 Assessment of sensory recovery

To evaluate the ingrowth of sensory nerves, the sensitivity to electrical stimulation of the skin was assessed weekly, starting from the first week postoperatively. In order to provoke a withdrawal reflex, a small electric current was applied locally to the skin of a conscious monkey, which had been fixed in a specially designed chair (Restraint Chair[R], Primate Products, CA, USA). A bipolar 50 Hz (sinusoidal) current source stimulator was used, delivering currents between 0.14 and 1.8 milliAmpere. The stimulation electrode consisted of two metal bars, each 1 mm in diameter and with a separation of 1.5 mm. The electrode was placed on the skin, at stimulation sites along three lines covering the transplant from distal to proximal (figure 1b). The contralateral side was used as a control. Stimulation sites were separated 0.5 cm along these lines. A continuous current of 1.8 milliAmpere (approximately 1.5 times the threshold for a withdrawal reflex at the contralateral hand) was delivered. The skin of the transplant was divided into distribution areas based on general human and rhesus monkey anatomical knowledge, in order to provide landmarks for consistent stimulation. The number of sites (range 30-40, depending on the size of the

replant) with positive withdrawal reactions related to the total number of measuring sites, provided the percentage of reinnervation.

C.2.5 *Assessment of motor recovery*

Motor reinnervation was evaluated by detection of Compound Motor Action Potentials (CMAP) of the thenar muscles after signal amplification with an electromyograph (Medelec[R], PN6). Signals were photographed from an oscilloscope. Under Ketamin (Fetelar[R]) anesthesia the median nerve was stimulated supramaximally, transcutaneously and proximal to the anastomosis, at elbow and wrist. Surface electrodes were placed over the belly of the abductor pollicis brevis muscle. At the same time a sensory potential was determined from the fifth finger to rule out inadvertent stimulation of the ulnar nerve. Latency (in a temperature controlled environment) and amplitude of CMAP of the thenar muscle in the transplanted- and contra-lateral side were compared weekly. Latency of CMAP was measured in milliseconds from stimululus artefact to the peak of the negative deflection. Amplitude of CMAP was measured in millivolts from the peak of the negative deflection to the baseline.

Sensory and motor reinnervation in the three replantation cases was evaluated monthly starting one year postoperatively. The twelve transplantation cases were evaluated weekly starting immediately postoperatively.

C.2.6 *Histology*

To examine nerve ingrowth, and thenar muscle reinnervation, the composite tissue grafts of three monkeys (two allografts and one autograft) were analyzed immunohistochemically. The opponens pollicis and the abductor pollicis brevis muscle in the thenar eminence of the operated as well as the contralateral hand were removed from origin to insertion and snapfrozen in liquid nitrogen chilled isopenthane. The presence and distribution of nerve bundles and functional motor end plates over the muscle were inspected microscopically. The detection of vital motor end plates was assessed using the histochemical method described by Karnovsky and Roots[13]. To detect the presence of axons within morphologically visible nerves, immunohistochemical staining with the monoclonal antibody 2F11[14] specific for neurofilaments was carried out, on formalin fixed paraffin embedded tissue.

C.2.7 Functional tasks

Video recordings of the operated hand of transplanted monkey no. 3439, and all replant cases, were made to evaluate overall function, whilst the animals were handling small particles of food.

C.3   Results
C.3.1   Graft survival

The three autologous replantations had complication free graft survival. The twelve successfully transplanted allografts had survival times that were short in six monkeys (21-33 days) and long in the other six (79-179 days). Ten out of twelve transplantations showed graft rejection. If macroscopical signs of rejection were observed, histological data were confirmative and revealed oedema, epidermolysis, dermal hemorrhage and lymphohistiocytic infiltrate. In five monkeys graft rejection was treated with the combination of monoclonal antibodies[11]. In two monkeys (4023 and 3439) rejection could be reversed successfully, in both monkeys on two occasions. In five other monkeys where graft rejection was treated with an increase in steroid dosage, reversal of rejection did not occur. The remaining two monkeys (3310 and 3308) had to be euthanized before rejection occurred, at 85 and 179 days postoperatively.
Other complications such as organ failure, lymphoid tumour development and death of the monkey are described elsewhere[15].

C.3.2 Sensory recovery

In the three monkeys in which an autologous replantation was performed, the percentage of positive withdrawal reactions of the median and superficial radial nerve skin area was 96%, when tested 2 years postoperatively.
In the allogeneic situation, six out of twelve allografts showed rejection before the return of sensibility could be demonstrated. The first signs of sensory recovery in the other six monkeys appeared after a mean of 41,8 days postoperatively (range 27-64 days; see table 1). The first positive withdrawal reaction occurred in the median nerve area in five monkeys, at the volar side of the thumb, just distal to the metacarpophalangeal joint, and in one monkey

at the level of the wrist. The percentage of median nerve reinnervation increased in time. The maximal sensory median nerve reinnervation for the six monkeys was 100, 91, 84, 80, 75 and 14% respectively (figure 2). In the monkeys with rejection of the transplant, longitudinal evaluation showed an immediate decrease in sensory recovery in monkey 4023 from day 55-62 and from day 104 onwards, in monkey 3439 from day 64 and in monkey 3992 from day 135. Rejection reversal showed a restoration of sensory recovery within a week in monkey 4023. In monkey 3439 the first sign of sensory recovery was demonstrated 10 days after reversal of a rejection episode (figure 2, table 1).

Table 1. First signs of functional recovery after transplantation.

Monkey	Motor	Sensory
3308	42	64
3310	56	29
3439	46	56[b]
4023	31	41
3992	<72[a]	34
2799	<79[a]	27

[a]first measurement with EMG
[b]rejection episode at day 41
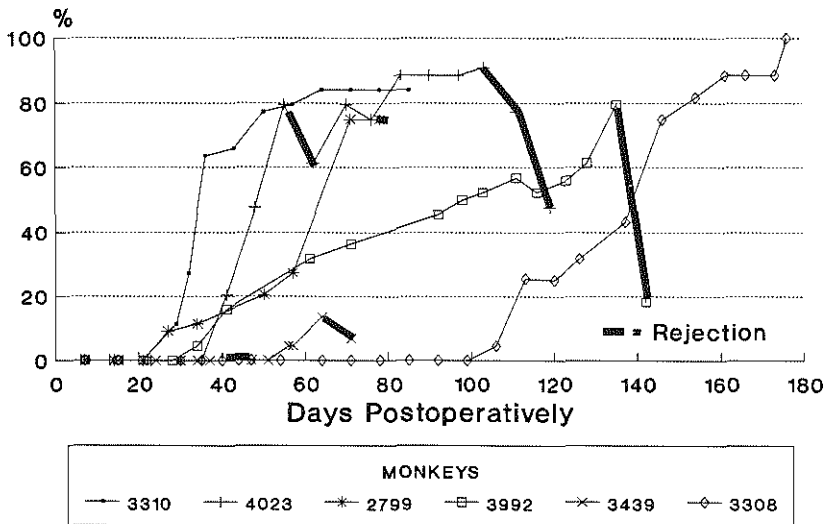
Figure. 2. Sensory reinnervation of the median nerve following radial hand transplantation in the rhesus monkey.
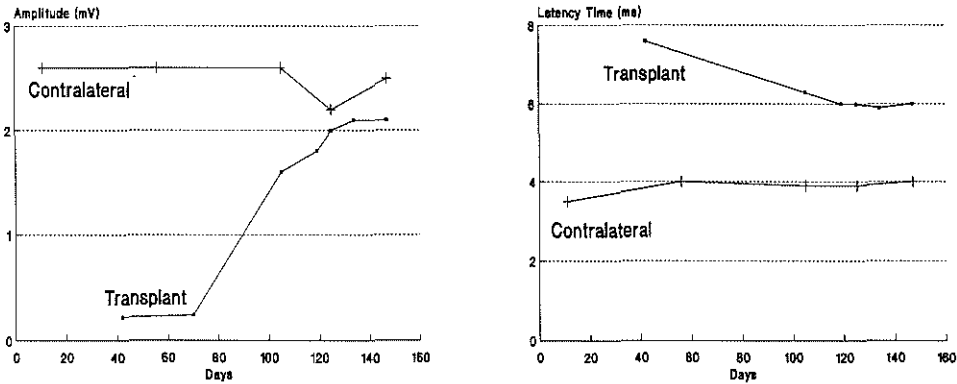
Figure 3a. The amplitude of the compound motor action potentials of the thenar muscles following stimulation of the median nerve at the elbow.
A comparison of the transplanted side to the contralateral side in monkey 3308.
Figure 3b. The distal thenar muscle latency time following stimulation of the median nerve at the elbow. A comparison of the transplanted side to the contralateral side in monkey 3308.

C.3.3 Motor recovery

In all long term survivors CMAP's could be measured. In four monkeys with an allograft, the first sign of motor recovery of the transplant could be detected after a mean of 43.8 days (range: 31-56 days). In two allograft recipients (2799 and 3992) for logistic reasons the electromyograph was first used at 72 and 79 days after transplantation respectively. At that time CMAP's of the thenar muscles of the allograft were present, so in these two monkeys the onset of motor recovery could not be established (see table I).

Amplitudes of CMAP's increased in time. The ratio of amplitudes of CMAP's of the thenar muscles in the three replanted monkeys was 88%, when compared to the amplitudes of CMAP's of the contralateral side one and a half year postoperatively. In two rejection free monkeys (3308 and 3310) evaluation of the amplitudes showed a similar pattern, following stimulation at the elbow as well as after stimulation at the wrist (figure 3a, monkey 3308). If rejection occurred, amplitudes of the CMAP's decreased. In monkey 3992, the amplitude of the CMAP decreased from 1.6 mV on day 128 to 0.9 mV on day 138 (a fall of 44%), while anti-rejection therapy was started on day 139 following transplantation.
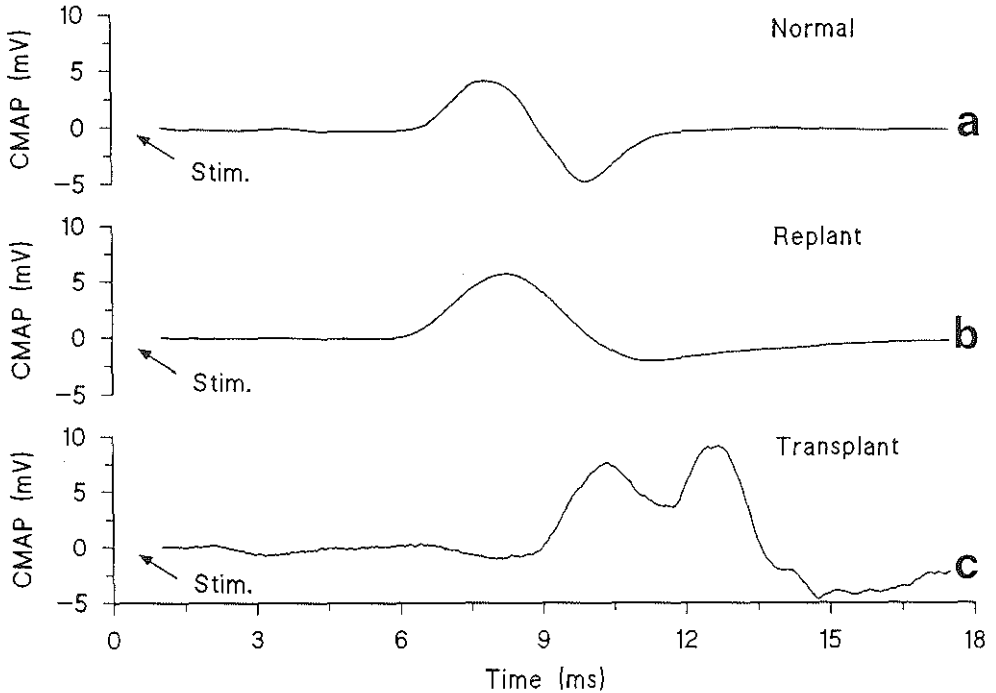
Figure 4a. CMAP (Compound Motor Action Potential) of the thenar muscles of the normal contralateral radial side of the hand of monkey TP. **Figure 4b.** CMAP of the thenar muscles of the replanted radial side of the hand of monkey TP. **Figure 4c.** CMAP of the thenar muscles of the allogeneic transplanted radial side of the hand of monkey 4023, recorded during the second rejection episode at day 121 postoperatively in a terminal experiment. All CMAP's of the thenar muscles are evoked by stimulation of the surgically exposed median nerve in the forearm at equal distances.

The amplitudes are comparable in all CMAP's. The onset of the CMAP's is comparable in (4a) and (4b). There is clearly delayed conduction indicating incomplete maturation in the median nerve of the transplant during the second rejection period (4c).

In monkey 4023, the amplitude decreased from 0,5 mV to 0,28 mV on day 83 (a fall of 44%), while anti-rejection therapy had started on day 62. After reversal of rejection, in this monkey the amplitude increased again to 0,5 mV on day 104, to decrease again to 0,4 mV (a fall of 20%) while the second anti-rejection therapy had started on day 111 after transplantation.

The ratio of the latency of CMAP of the thenar muscles in the three replanted monkeys was 93%, when compared to the contralateral side one and a half year postoperatively.

Latency time of CMAP decreased in relation to the time following transplantation (figure 3b, monkey 3308).

In a terminal experiment in the allograft recipient 4023 and in one replant, the median nerve was dissected and stimulated directly at the perineurium. During this assessment a CMAP of the thenar muscles could be recorded (figure 4).

C.3.4 Histology

The histological data confirmed actual thenar muscle re-innervation. Host nerve fibers clearly penetrated into both the auto- and allografts. Acetylcholine-esterase activity as evidence of vital motor end plates could be demonstrated in the thenar muscles of the investigated monkeys (figure 5).

Furthermore the presence of axons, more specifically neurofilaments, were demonstrated in the transplant, using immunohistochemical methods[14].

C.3.5 Function tasks

Videorecordings showed that in the replants as well as in the long term allograft recipients small particles of food could be picked up between thumb and index finger.

C.4 Discussion

In the present study functional recovery following allogeneic transplantation of the radial side of the hand was evaluated in twelve rhesus monkeys and in three monkeys following autologous replantation as controls. With regard to the allogeneic transplants, longitudinal follow-up assessed in conscious monkeys demonstrated sensory reinnervation in six monkeys. In the other six, early rejection made it impossible to test the return of sensation. The initial return of sensory reinnervation in the skin area corresponding to the median nerve occured at a mean of 41,8 days postoperatively (range : 27-64 days). If rejection occurred, an instant decrease was induced in the area in which a withdrawal reflex could be provoked. This is probably due to the fact that the first and primary target of the host immune response

against allogeneic composite tissue containing skin, appears to be the dermo-epidermal junction in which the sensory receptors are localized[3, 5, 6]. In the two cases where rejection could be reversed successfully with the monoclonal antibody therapy (figure 2, monkeys 4023 and 3439), the reduction or initial absence of sensory reinnervation was reversible. A subsequent reduction in reinnervation after a second episode of rejection in these monkeys prevented development of a recovery pattern comparable to that of the other monkeys with sensory reinnervation. This could imply that multiple rejection episodes impair complete sensory reinnervation, and confirm data presented by Samulack et al[6].

The order of sensory recovery in replanted digits is pressure, touch, pain, cold, warmth and perspiration[16]. Application of a small electrical current to the skin of the transplant mainly invokes pain sensation and probably some elements of pressure and touch sensation. Therefore the onset of sensory reinnervation is likely to be not the first sign of actual sensory reinnervation. The ingrowth of host axons into the allogeneic transplant, even under agressive immunosuppression reappeared with a mean of 42 days, over a distance of about 4 centimeters (anastomosis to tip of the thumb). In comparison the mean time of reappearance of sensation in clinically replanted digits following quillotine amputation at the proximal phalanx was 44 days[16]. Although the recovery times in allogeneic transplants and clinically replanted digits cannot be strictly compared it does give one an idea of the speed of recovery.

Longitudinal follow up demonstrated motor reinnervation in six monkeys with allogeneic transplants of the radial side of the hand, in the other six monkeys, early rejection prevented return of motor function. Initial return of thenar muscle reinnervation occurred after a mean of 43,8 days (range 31-56 days) postoperatively, measured by electromyography. Stark et al[4] reported active movement of the thumb 21 weeks and of the long fingers 22 weeks after operation. In our study after removal of the splint, movement of the thumb could be demonstrated in all six monkeys with long term graft survival, during tasks where food manipulation had to be carried out. However it could not be differentiated whether this thumb movement was mediated with or without assistance from thenar muscles. Dynamic electromyography during functional tasks could possibly solve this question[18].

The amplitude of the CMAP evoked in the allogeneic thenar muscles was decreased compared to the contralateral CMAP. The amplitude increased in time, postoperatively. This is in concordance with other studies concerning electrophysiological evaluation of muscle reinnervation after a nerve (crush)lesion [19, 20, 21]. The increase in amplitude in relation to postoperative time was possibly related to an increase in the number of regenerated axons and reinnervated muscle fibers. An increase in amplitude is proportional to the increase in the number of muscle fibers in the motor unit[22, 23].

The decrease of the amplitude of CMAP in the monkeys who showed rejection, suggests a

negative influence of rejection on the quality of motor recovery, especially monkey 4023 which showed a decrease in amplitude of CMAP at the time of the first rejection episode. The amplitude increased to original values again, following reversal of rejection, and before the second episode of rejection started. Further investigation will be needed to determine the influence of rejection on motor recovery of allografted muscles.

Gulati and Zalewski also, histologically demonstrated the presence of neuromuscular function in rat muscle allografts[24]. Immunohistochemical staining of sections of autologous and allogeneic grafts in our study yielded additional evidence for actual thenar muscle re-innervation. Vital motor-end plates could be demonstrated in the thenar muscles investigated (figure 5). Further evidence was retrieved from a terminal experiment in monkey (4023) where direct stimulation of the perineurium of the median nerve in the forearm produced CMAP at the heterologous thenar muscles (figure 4). These data are consistent with other primate studies[4, 5].

For the first time a longitudinal follow up of functional recovery following composite tissue allogeneic transplantation is presented. Clearly, there is substantial evidence of sensory and motor reinnervation of the allogeneic transplant by means of sensory measurements, electromyography (transcutaneously and intraoperatively),(immuno-)histology and the ability to perform specific motor tasks. It has to be taken into account, however, that improvement of motor and sensory function after nerve lesions or digital replantation may take years. Therefore, it can be expected that complication-free allograft survival for several years will allow an even better level of functional repair.

The complications which occurred in the monkeys due to the immunosuppressive regime varied from subclinical organ failure to lymphoid tumour development and death in seven out of twelve monkeys (submitted). Therefore significant immunological advancements will have to be made before heterologous transplantation of the upper extremity can be carried out in man. Further research will have to be done in the field of more specific and less toxic immunosuppressive agents.

Nevertheless it can be concluded that target tissues in composite allografts in non-human primates appeared to be re-innervated by host axons. The results for sensory and motor recovery, following allogeneic transplantation of the radial side of the hand, are from this point of view promising.

C.5 References

1. Black K.S., Hewitt C.W., Aniel M., Grisham G.R., Caiozzo V.J. and Achauer B.M. Neuromuscular Capabilities in Long-Term Composite Tissue Allografts. Transpl. Proc., vol. XX, no. 2, suppl. 2 (April), pp 269-271, 1988.
2. Press B.H.J., Sibley R.K., Shons A.R. Limb Allotransplantation in the Rat: Extended Survival and Return of Nerve Function with Continuous Cyclosporin/Prednisone Immunosuppression. Ann. Plast. Surg. no. 16, pp 313 - 327, 1986.
3. Daniel R.K., Egerszegi E.P., Samulack D.D., Skanes S.E., Dykes R.W. and Rennie W.R.J. Tissue transplants in primates for upper extremity reconstruction: A preliminary report. Journ. of Hand Surgery, Am. vol. 11a, no. 1: 1-8, 1986.
4. Stark G.B., Swartz W.M., Narayanan K., Möller A.R. Hand Transplantation in Baboons. Transpl. Proc. vol. 19, no. 5: 3968-3971, 1987.
5. Samulack D.D., Dykes R.W., Munger B.L. Neurophysiologic aspects of allogeneic skin and upper extremity composite tissue transplantation in primates. Transpl. Proc., vol. 20, no. 2, suppl. 2, 1988.
6. Samulack D.D., Munger B.L., Dykes R.W., Daniel R.K. Neuroanatomic evidence of reinnervation in primate allografted (transplanted) skin during cyclosporine immunosuppression. Neuroscience Letters, 72: 1-6, 1986.
7. Hovius S.E.R., Stevens H.P.J.D., Nierop P.W.M., Rating W., Strik R. van, Meulen J.C. van der. Allogeneic transplantation of the radial side of the hand in the rhesus monkey. I: technical aspects. Plast. Reconstr. Surg. (in press).
8. Napier J.R. Hands. London: George Allen & Unwin, 1980.
9. Warren J.M. Handedness of the Rhesus monkey. Science 118: 622-623, 1953.
10. McNeillage P.F., Studdert-Kennedy M.G., Lindblom B. Primate handedness reconsidered. Behavorial and Brain Sciences, 10: 247-303, 1987.
11. Stevens H.P.J.D., Hovius S.E.R., Nierop P.W.M. van, Rating W., Kwast Th.H. van der, Jonker M., Meulen J.C. van der. Immunological aspects of Partial Hand Transplantation in the Rhesus Monkey. Transpl. Proc. vol. 22, no. 4: 2006, 1990.
12. Stevens H.P.J.D., Roche N., Vuzevski V.D., Hovius S.E.R., Jonker M. In vivo additive immunosuppressive effects of monoclonal antibodies specific for CD3-, CD4-, CD8- and MHC class II antigens. Transpl. Proc., vol. 22, no. 4: 1783, 1990.
13. Karnovsky M.I., Roots L. A "direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem, no. 12: 219-221, 1964.
14. Klück P., Van Muyen G.N.P., Van der Kamp A.W.M. et al. Hirschsprungs disease studied with monoclonal anti-neurofilament antibodies on tissue sections. Lancet, 652-654, 1984.
15. Stevens H.P.J.D., Hovius S.E.R., Heeney J.L., Nierop P.W.M. van, Jonker M. Immunological aspects and complications of composite tissue allografting for upper extremity reconstruction: a study in the rhesus monkey. Transpl. Proc. (in press)
16. Yamauchi S., Nomura S., Yoshimura M., Ueno T., Iway Y., Shimamura K. A clinical study of the order and speed of sensory recovery after digital replantation. Journ. of Hand Surgery, 8 (5): 545-549, 1983.
17. Horch K.W. Absence of functional collateral sprouting of mechanoreceptor axons into denervated areas of mammalian skin. Experimental Neurology, 74: 313-317, 1981.
18. Cooney W.P., An K.A., Daube J.R. and Askew L.J. Electromyographic analysis of the thumb: a study of isometric forces in pinch and grasp. The Journal of Hand Surg. vol. 10A, pp. 202 - 210, 1985.

19. Ballentyne J.P., Campbell M.J. Electrophysiological study after surgical repair of sectioned human peripheral nerves. Journal of Neurology, Neurosurgery and Psychiatry, 36: 797-805, 1973.
20. Davis L.A., Gordon T., Hoffner J.A., Jhamandas J., Stein R.B. Compound action potentials recorded from mammalian peripheral nerves following ligation or resuturing. Journal of Physiology, 285: 543-559, 1978.
21. Buchtal F., Kühl V. Nerve conduction, tactile sensibility, and the electromyogram after suture or compression of peripheral nerve: a longitudinal study in man. Journal of Neurology, Neurosurgery, and Psychiatry, 42: 436-451, 1979.
22. Nandedkar S.D., Saunders D.B., Stalberg E.V. EMG of reinnervated motor units: a simulation study. Eletroencepehalography and clinical Neurophysiology, 70: 177-184, 1988.
23. Wee A.S., Ashley R.A. Where is the ideal reference site for recording the thenar muscle action potential. Electromyography and clinical Neurophysiology, 28: 249-252, 1988.
24. Gulati A.K., Zalewski A.A. Muscle allograft survival after Cyclosporin A immunosuppression. Exp. Neurol. 77: 378-385, 1982.

CHAPTER III: PARTIAL HAND TRANSPLANTATION
STUDY D : Complications of the employed immunosuppressive regimen

H.P.J.D. Stevens & S.E.R. Hovius

D.1 Introduction

Allotransplantation of a donor hand to a patient, lacking one or both hands due to trauma or congenital malformation, seems to become a more realistic alternative than it ever has been. Already, preclinical studies in nonhuman primates have been performed demonstrating the technical feasibility of transplantation of such a composite tissue allograft (CTA)[1-4]. Moreover, promising results were obtained with regard to sensory and motor function recovery of the CTA[5-7]. Reversal of rejection, even enabled renewed sensory reinnervation of CTAs within two weeks after onset of successful anti-rejection therapy with monoclonal antibodies (MAbs)[7]. However, the limiting factor that imposses reluctance upon actual hand transplantation in man, is the fact that the present immunosuppressive therapy can not ensure long-term complication free CTA survival. In previous studies in baboons, rejection occurred in three out of four[1] and six out of six[2] successfully transplantated allogeneic hands. In the latter study, one monkey died due to anesthesiological problems. Previously, we reported that ten out of 12 successfully transplanted allogeneic hands in the rhesus monkeys developed a rejection episode of their CTA[3,4,8]. Furthermore, seven out of 12 monkeys died during experiment.

This study focussed on the relation between the immunological aspects of allogeneic partial hand transplantation in the rhesus monkey, the case history of the monkeys involved and the complications encountered.

D.2 Materials and methods

D.2.1 Experimental model

In a preclinical model, transplantation of the partial hand was performed in adult rhesus monkeys (Macaca mulatta), as there is a close phylogenetic, anatomical, functional and immunological relationship between these monkeys and humans. CTA recipients were not alloimmunized prior to these experiments. However, in previous experiments three CTA recipients had donated a kindney and five monkeys had undergone experimental radiation of the testis as part of a previous unrelated procedure. Four monkeys had positive titers for Simian T-leukemia virus (STLV, Table 1). Another three monkeys became STLV-seropositive after blood transfusions or CTA acceptance from STLV positive donors.

Twelve technically successful allogeneic transplantations of the partial hand were performed in unrelated donor-recipient combinations, mismatched for rhesus major histocompatibility (RhLA) -A, -B and -DR antigens. The transplant consisted of the first ray

of the hand (the thumb) enlarged with a radial forearm flap of approximately 20 cm^2. Surgical aspects have been decribed previously[3,4].

D.2.2 Immunosuppressive treatment

Baseline immunosuppressive treatment incorporated daily subcutaneous administeration of 25 mg/kg Cyclosporine A (CyA) dissolved in Miglyol-812, starting one day before operation. Additionaly, an initial high dose of DAF (12 mg/kg/day) was administered for the first three postoperative days and tapered slowly until a maintenance dose of 1 mg/kg/day was reached after 12 days. If rejection was observed macroscopically (swelling, erythema, blister formation) and could be confirmed histologically (edema, hemorraghes, epidermiolysis, lymphohistiocytic infiltrates), then half of the group was treated by returning to the high dose (12 mg/kg/d) of DAF. Tapering of the DAF dose was done as described before. In the other half of the partial hand recipients, rejection was treated with a combination of MAbs, consisting of seven MAbs specific for CD3+, CD4+ CD8+ and MHC class II-DR positive cells as described previously[3,8]. All MAbs used in this study were crossreactive with rhesus lymphocytes, and were administered as an intravenous (iv) bolus injection for a period of ten days. Moreover, in this combination the cocktail of MAbs was capable to prolong skin allograft survival significantly to 19.3 (SD=1.8) days compared to a control group of 8.3 (SD=0.7) days[9].

Additionaly, the effect of preoperative third party blood transfusions was tested. Three blood transfusions, consisting of 20 ml of fresh whole citrated blood from donors that were fully mismatched with graft donors as well as graft recipients, were given at biweekly intervals before transplantation. The last transfusion was given 2 weeks before transplantation.

D.2.3 Monitoring and statistical analysis

CyA whole blood trough levels were measured with a specific radioimmunoassay (Sandimmun-Kit; Sandoz Ltd., Basle, Switzerland).

In the event of high morbidity or death, an complete autopsy was performed.

When appropriate, Fisher's exact-test, two sample t-test, single regression analysis and log-rank test were performed for hypothesis testing. Differences were considered significant if $P < 0.05$.

D.3 Results and discussion

Transplantation of the (partial) hand in the nonhuman primate is technically feasible[1-4] and results for functional recovery are promising[5-7]. In humans, however, such an operation can only be performed after many problems are solved first. Also ethical aspects require thorough attention. Most importantly, long term uneventful survival of a functional graft ought to be achievable.

Rejection of a CTA still is a major problem in the nonhuman primate. Various studies performed recently in this species, stressed the inevitablilty to use continuous high doses of CyA (20-48 mg/kg/day) to ensure long term allograft survival of CTAs[1,2,10]. However, in the baboon, using 20 mg/kg/day CyA and a maintenance dose of steroids, rejection occurred in three out of four[1] and six out of six[2] successfully transplantated allogeneic hands. Anti-rejection treatment with steroids could reverse one out of three and one out of six rejection episodes, respectively. In our study in the rhesus monkey, using 25 mg/kg/day CyA and a maintenance dose of steroids, ten out of 12 successfully transplanted allogeneic hands in the rhesus monkeys developed a rejection episode of their CTA. This happened, despite the fact that median CyA whole blood trough levels between day five and the day of onset of anti-rejection therapy ranged from 420 to 1253 ng/ml. Even 92% of all samples after day five was above the minimal required dose of 400 ng/ml[3]. Increased steroid treatment could not reverse allograft rejection (Table 1). MAbs therapy, however, did reverse CTA rejection in two out of five monkeys, and in both monkeys also a second rejection episode could be reversed. The log-rank tests revealed that if rejection of a CTA was treated with MAbs therapy, allograft survival times could be prolonged significantly longer than if than rejection was treated with a raise in steroids (P=0.015). This is in agreement with other clincal studies in which the MAb OKT3 (CD3-specific) was more effective in reversing rejection of allogeneic kidney and liver transplant, respectively[11,12]. Regarding these data, the minimal required CyA whole blood trough level to prevent CTA-rejection might even be higher than 400 ng/ml and anti-rejection therapy with immunosuppressive MAbs might, indeed, offer a better alternative for the treatment of rejection crises of CTAs, than increasing the steroid dose.

However, directly related to administration of multiple potent immunosuppressive drugs for a longer period of time, the occurence of side effects is increased. In our study, minor side effects of long term immunosuppressive therapy were primarily limited to weight loss due to anorexia. In the CTA-recipients that had graft survival longer than 70 days, weight loss ranged from eight to 40% (average 22%) in relation to preoperative weight.

Table 1. Allogeneic partial hand transplantation in the rhesus monkey; Case history, effects of Cyclosporine A, Prednisone and monoclonal antibodies on graft survival and encountered major complications[c].

Age	Kidney donor	Case history Testis radiation	α-STLV titer	Monkey	Protocol	Rejection reversal	Graft survival times (days)	Cause of death
15[a]	+	-	+	2799	MAbs	-	79[c]	shock
6[a]	-	+	sc	4023	&	+		
					transf.	+[b]	121[c]	sepsis/PTLP-disorder development
14[a]	-	+	sc	2988		-	22[c]	PTLP-disorder
19	+	+	-	3992	MAbs	-	144	
13[a]	-	-	lt	3439		+		
						+[b,d]	97[c]	PTLP-disorder
13[a]	-	+	-	3308		no rejection	179[c]	PTLP-disorder
15[a]	-	-	+	2596	DAF	-	30	
13	+	-	-	2I	&	-	29[c]	sepsis
14[a]	-	+	nt	3310	transf.	no rejection	85[c]	sepsis
7	-	-	sc	1FU	DAF	-	33	
19[a]	-	-	+	1550		-	33	
14[a]	-	-	+	3212		-	21	

[a]jungle caught monkeys, approximately 2-5 years older than tabulated; [b]second rejection episode; [c]death, spontaneously or due to euthanasia; [d]reversal of rejection was partial: skin on the palmar side of the thumb and thenar was rejected.
Abbreviations: **STLV**: Simian T Leukemia Virus; **sc**: seroconversion; **MAbs**: anti-rejection therapy consisted of a ten day course of a combination of seven monoclonal antibodies; **transf.**: three third party blood transfusions were given to the recipient, preoperatively; **DAF**: anti-rejection therapy implied a raise in steroids; **lt**: low titer; **nt**: not tested; **PTLP**: posttransplantation lymphoproliferative (disorder).

During immunosuppressive treatment, levels of creatinine were elevated in five monkeys; levels of lactate dehydrogenase and aspartate transaminase were raised in three monkeys and in four other monkeys liver function parameters were elevated incidentally. Nevertheless, no severe nephro- and/or hepatotoxicity were observed. Using similar high doses of CyA and DAF, more severe kidney and liver damage may occur in the humans[13]. Gingival hyperplasia, hypertrichosis and neurotoxicity have not been observed.

Major side effects of the immunosuppressive therapy concerned the death of seven monkeys, during experiment (Table 1). One monkey died due to an irreversible shock directly after the first injection of MAbs. Anti-CD3 and anti-CD8 MAbs are known to, incidentally, induce shock-like side effects directly after administration. Most likely these effects are related to stimulation of T cells to produce lymphokines and/or to the rapid cell clearance[14]. Possibly, temporary addition of MAbs specific for lymphokines such as tumor necrosis factor-alpha, which is known for its important role during lymphokine-related shock[15], might reduce the threat of this particular side effect. Three monkeys died due to opportunistic bacterial infections, 29, 85 and 121 days after operation. Three others died from multiple organ failure, due to the presence of posttransplantation lymphoproliferative (PTLP)-disorders, 22, 97 and 179 days after transplantation. In a fourth monkey that died due to sepsis, also PTLP-disorder development was observed at autopsy.

Overall, no correlation between CyA whole blood trough levels and occurrence of death either by sepsis or PTLP-disorder was present (t-test, $P > 0.05$). Similar doses of CyA and DAF were administered for periods over one year in baboons and no such side effects were reported[1,2]. Addition of MAbs anti-rejection therapy to baseline immunosuppression did not correlate significantly to occurrence of death ($P > 0.05$, Fisher test).

Though our population is small, an enhanced predisposition in the monkeys that received MAbs anti-rejection therapy is found to PTLP-disorder development ($P = 0.03$, one-sided Fisher test). It might be a consequence of addition of a third potent immunosuppresive agent to baseline therapy[16]. On the other hand, the used MAbs were specifically aimed at lymphocytes, which might have precipitated lymphoid lesions, rather than other malignancies. Alternatively, virus infections, notably Epstein-Barr[17] and HTLV-virus infections[18], have a possible etiologic link with the development of lymphoma. In some CTA recipients, serum antibody titers against STLV have been demonstrated (Table 1). However, no significant correlation occurred between the presence of anti-STLV titers and lymphoma development ($P > 0.05$, Fisher test).

Neither did other historic burdens like kidney donorship or testis radiation correlate to occurrence of death in general nor death by spesis or PTLP-disorders nor PTLP-disorder development ($P > 0.05$, Fisher test).

In conclusion, a more effective, less toxic immunosuppressive regimen is needed before actual allogeneic transplantation of the hand should be performed in human patients, though it was demonstrated previously that it is technically feasible and that results for functional recovery are promising.

D.4 References

1. Daniel RK, Egerszegi EP, Samulack DD, et al: J Hand Surgery 11:1, 1986
2. Stark GB, Swartz WM, Narayanan K, et al: Transplant Proc 19:3968, 1987
3. Stevens HPJD, Hovius SER, Vuzevski VD, et al: Transpl Proc 22:2006, 1990
4. Hovius SER, Stevens HPJD, Nierop PWM van, Rating W, Strik R van, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. I. Technical aspects. J Plast Recon Surg (accepted for publication).
5. Samulack DD, Munger BL, Dykes RW, et al: Neuroscience Lett 72:1, 1986
6. Narayanan K, Swartz WM, Stark B, et al: Transpl Proc 20: 335, 1988
7. Hovius SER, Nierop PWM van, Stevens HPJD, Godschalk M, Kusuma A, Mechelse K, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. II. Functional aspects. J Plast Recon Surg (accepted for publication).
8. Stevens HPJD, Hovius SER, Kwast ThH van der, et al: Composite tissue allografting: Effects of cyclosporine A, prednisone, blood transfusions and monoclonal antibodies. Transplantation (submitted).
9. Stevens HPJD, Roche N, Hovius SER, et al: Transpl Proc 22: 1783, 1990
10. Skanes SE, Samulack DD, Daniel RK. Transpl Proc 18:898, 1986
11. Starzl TE, Fung JJ. Transpl Proc 18:937, 1986
12. Ortho Multicenter Transplant Study Group. New Eng J Med 313:337, 1985
13. Scott JP, Higenbottam TW. Med Toxicol 3:107, 1988
14. Jonker M, Nooij FJM, den Butter G, et al: Transplantation 45:667, 1988
15. Tracey KJ, Fong Y, Hesse DG, etal: Nature 330:662, 1987
16. Penn I. Transplantation 43:32, 1987
17. Hanto DW, Gajl-Peczalska KJ, Frizzera G, etal: Ann Surg 198:356, 1983
18. Clark JW, M Robert-Guroff, Ikehera O, etal: Cancer Research 45:2849, 1985

CHAPTER III: PARTIAL HAND TRANSPLANTATION

STUDY E   : Development of STLV-provirus positive lympho-
              proliferative disorders in rhesus monkeys
              immunosuppressed for transplantation

H.P.J.D. Stevens

Submitted; Laboratory investigation

E.1 Introduction

Various types of lymphoproliferative disorders have been described in human transplant patients following transplantation in the presence of Cyclosporine A (CyA) and Prednisone immunosuppression[22,25,28,34]. A previous study noted a strong association of proliferative lymphoid lesions in transplant patients with Epstein-Barr virus (EBV) and a spectrum of lesions ranging from those of mononucleosis to malignant lymphoma[10,12]. An additionally important virus for consideration is HTLV-1, the etiologic agent of an aggressive form of adult T-cell lymphoma, frequently described in southern Japan[9] and the Caribean[3a]. Lymphoma in man associated with HTLV-1 commonly presents with cutaneous lesions, hypercalcemia and has a mature T-cell phenotype but variable morphologic appearance[33]. The incidence of lymphoma in normal healthy individuals infected with HTLV-1 is low. Approximately less than 1% of HTLV-1 infected people are believed to develop cancer under normal conditions over an entire lifespan although the virus transforms cells *in vitro*[9,18]. HTLV-1 has also been demonstrated to transform monkey lymphocytes[19]. In most primate species HTLV-like viruses, designated Simian T-cell leukemia viruses (STLV) occur with great frequency in large numbers of old world primates. Those strains which have been sequenced have been found to have more than 90% sequence homology and have marked similarity in genomic structure and morphology[7,13,24,35,37,38]. These viruses are very prevalent and occur naturally in a great number of primate species[5,6,11,14,17,20,21,39]. A spectrum of lymphoproliferative diseases have been described in a variety of STLV infected non-human primates[1,16,23,26,27,36]. In captive colonies of non-human primates the seroprevalence can be greater than 30 percent[11,17].

In this study in the Macaca mulatta the role of STLV during the development of post-transplantation lymphoproliferative (PTLP) disorders was investigated.

E.2   Materials and methods
E.2.1 Clinical setup of the experiments

Strong immunosuppressive therapy was instituted to prevent rejection of allografts in 12 mature outbred rhesus monkeys (Macaca mulatta) having received a successfully transplanted allogeneic radial side of the hand[30]. The immunosuppressive regime consisted of a daily maintenance dose of 25 mg/kg daily subcutaneous doses of CyA dissolved in Miglyol-812, starting one day preoperatively. An initial high dose of steroids (Di-Adreson-F$_{aquosum}$ = DAF, 12 mg/kg/day) was administered (im) for the first three postoperative days and tapered slowly until a maintenance dose of 1 mg/kg/day was reached after twelve days.

If rejection was observed macroscopically (swelling and erythema) and could be confirmed histologically, the animals were treated by increasing the immunosuppressive regime. Six animals were treated by an increase of the DAF dose to 12 mg/kg/day, followed by tapering of the DAF dose as described above. In the other six allograft recipients, rejection was treated with a combination of MAbs, administered as an (iv) bolus injection for a period of ten days. This cocktail of MAbs consisted of B8.12 and 7.5.10.1 both specific for MHC class II-DR antigens, 1 mg/kg each; 5B11 and FN18, both CD3-specific; RIV6 and RIV7, both CD4-specific; and FK18, specific for CD8+ cells, the latter 5 MAbs were given in a dose of 0.5 mg/kg[32]. All MAbs used in this study were crossreactive with rhesus monkey lymphocytes.

The therapeutic value of these rejection treatment protocols was tested, in combination with preoperative third party blood transfusions. Six animals received three third party blood transfusions, consisting of 20 ml of fresh whole citrated blood from random donors, were given at biweekly intervals before transplantation. The last transfusion was given 2 weeks before transplantation.

Following successful surgery, seven animals became terminally ill during the post-transplantion period. They were euthanized and a complete autopsy was performed. Tissues were fixed in 10% neutral buffered formalin and examined by routine histochemistry. When possible fresh tissues including, lesions, draining lymph nodes and graft sites were quickly frozen in liquid nitrogen for further analysis.

E.2.2 Immunohistochemical characterization of PTLP-disorders

The presence or absence of PTLP-disorders was confirmed histologically and classified morphologically according to the NCI working formulation. For this purpose, tissue sections harvested at autopsy were fixed in buffered formalin and processed routinely for histology on hematoxylin and azofloxine (H/A) stained sections. Parallel biopsy material was snap-frozen in liquid nitrogen chilled isopentane and used for immunohistochemical studies. Tissue bound primary antibody was reacted with horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS containing 5% heat-inactivated normal rhesus serum and 5% normal rabbit serum. Conjugate binding was visualized with a solution of 0.5 mg/ml 3,3,-diaminobenzidine tetrahydrochloride (Fluka Chemika, FRG) and 0.03% H_2O_2 in PBS. The following MAbs were used: T11, specific for CD2 (Dakopatts, Glostrup, Denmark), OKT4 specific for CD4, (Ortho Diagnostic Systems, Raritan N.J.), FK18 specific for CD8, (Dr F. Koning, Bloodbank, University of Leiden, The Netherlands), A-K specific for k-light chain , A-L specific for λ-light chain, (Jonker M, Primate

Center-TNO, Rijswijk, The Netherlands) anti-MHC class II specific for MHC class II antigens (Becton Dickinson, Sunnyvale CA) and the MAb Ki67, (Dakopatts) specific for a proliferation associated nuclear antigen (Chapter II, study E). To control for possible background staining, incubations of primary antibody were omitted in control slides of each tissue.

E.2.3 Serological assessment HTLV-1 related virus infections

Previous screening of the rhesus colony for various viruses had indicated a high prevalence of STLV (30-40% of the colony, J.L. Heeney, 1989 Annual Report ITRI-TNO), a simian retrovirus found in a wide range of non-human primate species. HTLV-1 enzyme-linked immunoenzyme assays (ELISA) were commercially obtained (Du Pont de Nemours International S.A., Geneva) and tested for cross reactivity with STLV using positive and negative serum from colony infected monkeys. This test was used to screen monkeys involved in the experiments. To further confirm ELISA results and the relatedness of these viruses to HTLV-1, various serum samples found to be ELISA positive were tested for antigenic similarity on HTLV-1 western blots and compared the results with serum from HTLV-1 infected patients.

Serum samples were obtained from the rhesus monkey colony at TNO Rijswijk and from animals in this study from time points; 1) prior to pre-operative third party blood transfusions, 2) following transfusions but before transplantation and, 3) several months post-transplantation.

E.2.4 Assessment of HTLV-1 like provirus infection at DNA level

When sufficient tissues were available following phenotypic analysis, remaining tissue was used to prepare DNA for southern blot analysis.

DNA was extracted from PTLP-tissue by lysis in 20 ug proteinase K, 0.5% sarcosyl solution, incubated at 50 °C and extracted at least twice in PCIA (phenol, chloroform, isoamylalcohol, 24:24:1). The aqueous phase was then centrifuged in a CsCl step gradient. DNA was dialyzed against 10mM Tris-HCl, 1mM EDTA, pH 8.0, ethanol precipitated and digested with Hind III. After electrophoresis and 4 M NaOH transfer to Biotrace nylon blotting medium the DNA was probed with full length, 32P labeled 9 kb full length (minus LTRs) pCS-HTLV-1 clone (D. Derse, NCL, Frederick MD) or partial fragments consisting of the envelope or tax regions and hybridized in 50% formamide, 10X Denhardt reagent, 100 ug of human placental DNA, 0.2% SDS, and 6X SSC for 48 hours. Blots were washed for 1 hour

in 1X SSC at 25°C, 1 hour in 1X SSC at 37°C and 0.1X SSC at 50°C.

E.2.5 Statistical analysis

When appropriate, Fisher's exact-test, two sample t-test and log-rank test were performed for hypothesis testing. Differences were considered significant if $P < 0.05$.

E.3   Results

E.3.1 Findings at necrospy

Following immunosuppressive therapy for allogeneic transplantation of the radial side of the hand[30], seven monkeys died during the experimental period (Table 1). In the remaining five other monkeys, the experimental protocol was continued untill an irreversible immune response had completely rejected the allograft. Detailed information on technical, immunological and functional aspects of these experiments is described elsewhere[12,30] (Chapter III, study A,B,C).

In the group of transplant recipients that had received pre-tansplant blood transfusions and was treated with MAbs-anti-rejection therapy if neccessary, one monkey (#2799) died due to an irreversible shock directly after the first administration of MAbs, 79 days postoperatively. Monkey #4023 that twice received MAb therapy died due to spesis, 10 days after onset of the second episode of graft rejection. At autopsy of this monkey, evidence of wide-spread lymphoproliferation was observed. Monkey #2988 died due to multicentric PTLP-disorder 22 days after operation. At this time the allograft was fully rejected despite seven days of MAbs administration[30].

In the group of transplant recipients that had not received pre-tansplant blood transfusions but was treated with MAbs-anti-rejection therapy if indicated, one monkey (#3439) died due to a malignant follicle center cell PTLP-disorder, 22 days after onset of the second episode of rejection treatment with MAbs. Another monkey (#3308) died before rejection had occurred from a multicentric PTLP-disorder 179 days postoperatively.

Complications in the group of transplant recipients that recieved pre-transplant blood transfusions and wich were treated with an increase in DAF if allograft rejection occurred were as follows; one monkey (#2I) died due to sepsis 29 days postoperatively, monkey #3310 suffered from sepsis 85 days following transplantation. Primary sites of infection were a skin phlegmone and a skin abces, respectively.

Table 1. Allogeneic transplantation of the radial side of the hand in the rhesus monkey:
anti-STLV titers in serum specification, duration of
administered immunosuppressive therapy, and cause of death.

Monkey	anti-STLV titer	Protocol	Duration of basic immunosuppressive therapy (days)[a]	Duration of anti-rejection therapy (days)	Cause of death
2799	+	MAbs	79[b]	1x1[c]	shock
4023	sc	&	121[b]	2x10	sepsis/PTLP-disorder
2988	sc	transf.	22[b]	1x7[c]	multicentric PTLP-disorder
3992	-	MAbs,	144	1x4[c]	-
3439	lt	no transf.	97[b]	2x10	follicle center cell PTLP-disorder
3308	-		179[b]	-	multicentric PTLP-disorder
2596	+	DAF	30	1x12	-
2I	-	&	29[b]	1x8[c]	sepsis
3310	nt	transf.	85[b]	-	sepsis
1FU	sc	DAF,	33	1x12	-
1550	+	no transf.	33	1x12	-
3212	+		21	1x9[c]	-

[a]Daily maintanance doses of immunosuppression consisted of 25 mg/kg CyA and 1 mg/kg DAF; [b]death, spontaneously or due to euthanasia; [c]The proposed duration of anti-rejection therapy (10 days for MAb-therapy and 12 days for DAF-therapy) was not completed.
Abbreviations: STLV: Simian T Leukemia Virus; **PTLP-disorder:** post-transplantation lymphoproliferative disorder; sc: seroconversion; lt: low titer; nt: not tested; MAbs: anti-rejection therapy consisted of a ten day course of a combination of seven monoclonal antibodies; **transf.:** three third party blood transfusions were given to the recipient, preoperatively; **DAF:** anti-rejection therapy consisted of a raise in steroid treatment.

In the last group of transplant recipients that did not receive pre-tansplant blood transfusions but was also treated with DAF-anti-rejection therapy if indicated, no monkeys died during experiment. However, it should be noted that allograft survival times in this group were shorter than in the treatment groups and thus received immunosuppression for a shorter period of time.

In the four monkeys which were found to have PTLP-disorders, all lymphoproliferation was multicentric with varriable morphologic characteristics ranging from a lymphoreticular to follicular center cell morphology.

E.3.2 Influence of immunosuppressive treatment and case history

Addition of MAbs therapy to baseline immunosuppression did not have a significant effect on occurrence of death in general (P > 0.05, Fisher's exact-test)[29]. Though the population treated was small, an enhanced predisposition to PTLP-disorder development (P = 0.03, one-sided Fisher's exact-test) was noted in monkeys which received MAb anti-rejection therapy, possibly as a consequence of additional, potent immunosuppression in addition to baseline therapy[25]. Other factors in the case history of each animal like age, sex, CyA whole blood trough levels, kidney donorship and experimental low-dose radiation of the testis were examined for possible correlation with PTLP-disorder development but were found to be non-significant (P > 0.05, Fisher's exact-test)[29] (ChIII; study D).

E.3.3 Relevance of anti-STLV titers to PTLP-disorder development

All animals were tested for the presence of anti-STLV antibodies before, during and after experiment. Four animals were seropositive before experiment. Four animals aquired anti-STLV antibodies in the course of the experiment (Table 1). In each case of PTLP-disorder development, STLV-provirus was aquired during the experimental period.

This was clearly evident serologically in two of these animals, and a third which had developed a low titer shortly before death (Table 2). In one other animal (#1FU), evidence of virus infection was also observed in the course of experiment but this animal has not developed tumor or any other complications to date. Of the seven animals which died in the experimental period for reasons other than PTLP-disorders, only one animal (#2799) was STLV seropositive. Retrospective testing demonstrated that this monkey was infected prior to the experiment and died of shock, presumably as a result of the MAb therapy[29].

Serologically, virus transmission could be traced to have come from STLV-positive monkeys, either by preoperative blood transfusions or by allograft donation through demonstration ofseroconversion for STLV titers in transplant recipients.

Animals which retrospectively had serologic evidence of STLV infection prior to the experiment (n = 4), did not develop PTLP-disorders sugesting that active STLV infection during immunosupressive therapy was an important factor in PTLP-disorder development.

Table 2. Detectable antibody titers against Simian T-Leucocyte Virus (STLV) in four rhesus monkeys with PTLP-disorders after transplantion of the radial side of the hand; relation to the administration of pre-operative blood transfusions and transplantation[a].

Time Interval	Monkey #4023	Donors[a] Graft	Blood	Monkey #2988	Donors[a] Graft	Blood	Monkey #3439	Donor[a] Graft	Monkey #3308	Donor[a] Graft
before Tx	-	+	+	-	nt	-	-	nt	-	-
after transf. before Tx	+			-			_		-	
after Tx	+			+			lt		-	

[a]Monkeys that were fully mismatched for MHC class I and II antigens of the graft recipient as well as the graft donor, donated three 'third party' blood transfusions that were given to the graft recipient at biweekly intervals at -6, -4 and -2 weeks before the day of operation. On the day of operation the allogeneic radial side of the hand was transplanted from a fully mismatched graft donor to the transplant recipient. **Abbreviations: Tx:** transplantation; **nt:** not tested; **lt:** low titer.

E.3.4 Immunohistochemical staining of lymphoproliferative tissue

Frozen sections of lesions collected at necropsy were assessed for phenotypic characteristics using the following pannel of phenotypic markers; CD2, CD4, CD8, k-light chain, λ-light chain, MHC-DR and the proliferation marker Ki67. In each case the staining patern varried and was frequently complicated by mixed population of residual benign leukocytes. All cases stained strong positive for the MHC class-II DR marker and a small mixed population of light chain positive cells were frequenty seen in each case. In the frozen tissues available to us to study there were not sufficient sections of homogenous lymphoproliferative tissue to make a conclusive statement of the PTLP-disorder phenotype.

E.3.5 Evidence of STLV infection at the DNA level

Confirmatory evidence of STLV infection was determined by southern blot analysis of rhesus DNA extracted from available PTLP-tissue. Furthermore, this technique was used to attempt partial characterization of the HTLV-1 like virus.
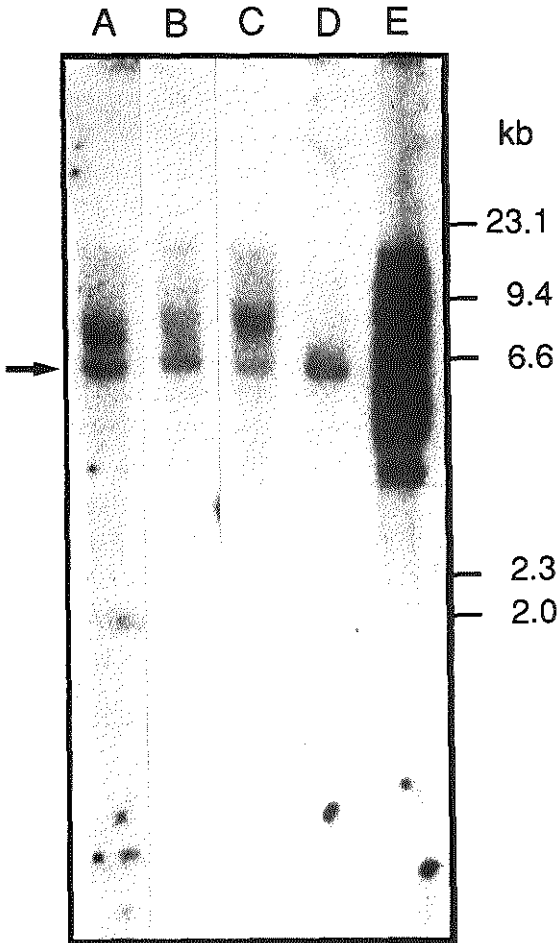
Figure 1.
Southern blot analysis of DNA, extracted from post-transplantation lymphoproliferative tissue of rhesus monkeys #4023 (lane A), #2988 (lane B), #3439 (lane C) and #3308 (lane D). Lane E concerns DNA extracted from the HTLV-1 tumor derived cell MT-2 containing multiple provirus copies. The blot was probed with the HTLV-1 probe PCS-HTLV-1 minus the long term repeats. The left hand arrow indicates a common 5.4 Kb band in rhesus lymphoid tissue which hybridizes with the HTLV-1 pobe.

It was demonstrated that STLV-provirus was present in all four PTLP-disorder cases by southern blot (Fig. 1). Using partial HTLV-1 fragments as probes we confirmed that under conditions of high stringency the rhesus STLV had envelope, polymerase and gag regions were highly homologous with HTLV-1. We were not able to consistently demonstrate hybridization of the Tax region of HTLV-1 with rhesus tumor DNA samples suggesting that differences in this region exist at the molecular level.

E.4 Discussion

This study identified several important problems concerning selection of transplantation donors and recipients and has identified, for the first time, the oncogenic potential of STLV in rhesus macaques. Four of seven animals which died following allogeneic transplants of the radial side of the hand developed lymphoproliferatiev disorders which contained STLV-provirus. In two of the four cases which developed lymphoid lesions following immunosupressive therapy we were able to demonstrate serologically that STLV was acquired from blood or organ donor. In another case which became seropositive post-transplantation the serologic status of the donor was not available. One monkey did not receive blood transfusions and after acceptance of a graft from a seronegative donor, never developed titers to STLV, but had STLV-provirus demonstratable in PTLP-tissue tissue. These two cases with low or undetectable STLV titers suggest that infection was acquired during the period of intensive post-transplantation immunosupressive therapy and that detectable HTLV-1 cross-reacting antibodies did not develop to significant titers during this treatment and prior to PTLP-disorder development. This implicates that infection might also be possible via other routes than blood or an allograft. In this respect it is interesting to note that monkeys in this study were housed four by four in separate cages in the same room during experiment.

The morphologic and phenotypic characteristics of these PTLP-disorders in macaca mulatta varied significantly. Furthermore, insufficient sections of homogeneous tissue were available making it difficult to assign a specific phenotype to the lymphoprolferative cells. If PTLP-disorder development in this species infected with STLV is analogous to HTLV-1 associated lymphomas in man, then one would expect to identify a mature CD4 positive T-cell phenotype[33]. This was not apparent in this study suggesting that the rhesus STLV causes PTLP-disorders of a more diverse cell type.

In a recent study by Swinnen et al[32a] addition of OKT3 (an anti-CD3 MAb) to the immunosuppressive regimen increased the incidence of PTLP-disorder after clinical cardiac transplantation. Furthermore, a higher frequency of primary infection with EBV occurred in the group of patients that had lymphoid tumor development. However, there appeared not to be a causal relation between these two phenomena. Thus in concordance with previous reports primary EBV infection had been identified as carrying a higher risk than reactivated infection in the development of PTLP-disorders, but also anti-CD3 in itself does seem to precipitate lymphoid tumor development[12]. In our study the finding that MAbs-anti-rejection therapy showed an enhanced predisposition to PTLP-disorder development (P = 0.03, one-sided Fisher's exact-test) coincided with the fact that in each case of PTLP-disorder development, STLV-provirus was acquired during the experiment. This might indicate that two different mechanisms

both increased the chance of lymphoid proliferation, resulting in the variable morphologic characteristics of these disorders.

Furthermore, it should be noted that PTLP-disorders in this study arose during heavy cyclosporine A treatment, which by itself can cause, or permit tumor development in heavy immunosupressive protocols[2,3,4,15,22]. Recently it has been described that cyclosporine A acts through nuclear protiens involved in T-cell activation[7] some of which interact with the HTLV-1 tax gene suggesting a mechanism in which T-cell cancer can develop.

This relationship between CyA and MAb (anti-CD3) therapy, HTLV-1 (STLV-1) infection and PTLP-disorder development in rhesus monkeys indicates the possible clinical risk of treating HTLV-1 infected patients with an immunosuppressive protocol which include these drugs. Based on the findings in this transplantation model in the <u>Maccaca Mulata</u>, the importance of screening blood or organ donors for HTLV-1 must be emphasized.

E.5 References

1. Blakeslee, J.R., McClure, H.M., Anderson, D.C., Bauer, R.M., Huff, L.Y. and Olsen, L.Y., Chronic fatal disease in Gorillas seropositive for simian T-lymphotropic virus I antibodies. Cancer Lett., Oct. 37 (1), 1-6 (1987).
2. Borel, J.F., Cyclosporine: Historical perspectives. Transplant. Proc. 15, 2219-2229 (1983).
3. Bunjes, D., Hardt, C., Rollinghoff, M. et al., Cyclosporin A mediates immunosuppression of primary cytotoxic T-cell responses by impairing the release of interleukin 1 and interleukin 2. Eur. J. Immunol. 11, 657-661 (1981).
3a. Clark, J., Saxinger, C., Gibbs, W.N., Lofters, W., Langrande, L., Deceular, K., Ensroth. A., Robert-Guroff, M., Gallo, R.C., Blattner, W.A., Seroepidermiologic studies of human T-cell leukemial lymphoma virus type I in Jamaica. Int. J. Cancer 36, 37-42 (1985).
4. Cleary, M.L., Warnke, R., Sklar, J., Monoclonality of lymphoproliferative lesions in cardiac transplant recipients. N. Engl. J. Med. 310, 477-482 (1984).
5. Coursaget, P., Barres, J.L., Yvonnet, B., Chiron, J.P., Cornet, M., Ferrara, L. and Eyraud, M., Antibodies to human T-cell leukemia virus (HTLV-1) in non-human primates from Senegal. Biomed. Pharmacother. 39, 198-199 (1985).
6. Dracopoli, N.C., Turner, T.R., Else, J.C., Jolly, C.J., Anthony, T., Gallo, R.C. and Saxinger, W.C., STLV-I antibodies in feral population of East African Vervet monkeys (Cercophithecus aethiops). Int. J. Cancer 83, 523-529 (1986).
7. Emmel, E.A., Verwey, C.L., Durand, D.B., Higgins, K.M., Lacy, E. and Crabtree, G.R., Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science 246, 1617-1620 (1989).
8. Guo, H.G., Wong-Staal, F. and Gallo, R.C. Novel vrial sequences related to human T-cell leukemia virus in T cells of a seropositive baboon. Science 223, 1195-1196 (1984).
9. Hanaoka M: Clinical pathology of adult T-cell leukemia, Acta Haem Japanese 44:1420, 1981.
10. Hanto DW, Gajl-Peczalska KJ, Frizzera G, Arthur DC, Balfour HH, McClain K, Simmons RL, Najarian JS. Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occurring after renal transplantation. Clinical, pathological, and virological findings and

implications for therapy. Ann Surg 198:356-69 (1983).

11. Hayami, M., Komuro, A., Nozawa, K., Shotake, T., Ishikawa, K.I., Yamamoto, K., Ishida, T., Honjo, S. and Hinuma, Y., Prevalence of antibody to adult T-cell leukemia virus-associated antigens (ATLA) in Japanese monkeys and other non-human primates. Int. J. Cancer 33, 179-183 (1984).

12. Ho, M., Miller, G., Atchison, R.W., Breinig, M.K., Dummer, J.S., Andiman, W., Starzl, T.E., Eastman, R., Griffith, B.P., Hardesty, R.L., Bahnson, H.T., Hakala, T.R. and Rosenthal, J.T., Epstein-Barr virus infection and DNA hybridization studies in post-transplant lymphoma and lymphoproliferative lesions: The role of primary Infection. J. Infect. Dis. 152, 876-886 (1985).

13. Komuro, A., Watanabe, T., Miyoshi, I., Hayami, M., Tsujimoto, H., Seiki, M. and Yoshida, M., Detection and characterization of Simian retroviruses homologous to human T-cell leukemia virus type 1. Virology 138, 373-378 (1984).

14. Kubonishi, I. and Minezawa, M., Detection of type C virus particles in Japanese monkeys seropositive to adult T-cell leukemia-associated antigen. GANN 73, 848-849 (1982a).

15. Lafferty, K.J., Borel, J.F. and Hodgkin, P., Cyclosporine-A (CsA): Models for the mechanism of action. Transplant. Proc. 15, 2242 (1983).

16. Lapin, B.A., Iakavleva, L.A., Voevodin, A.F., Indzhiia, L.A. and Agrba, V.Z., Study of virus-associated lymphomas in primates using a model of malignant lymphoma of the baboon. Vopr.-Onkol. 29 (12), 61-66 (1983).

17. Lowenstine, L.J., Pedersen, N.L., Higgens, J., Pallis, K.L., Kyeda, A., Marx, P., Lerche, N.W., Munn, R.J. and Gardner, M.B., Seroepidemiologic survey of captive old-world primates for antibodies to human and Simian retroviruses , and isolation of Lentivirus from Sooty Mangabeys. Int. J. Cancer 38, 563-574 (1986).

18. Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K., Hinuma, Y., Type C virus particles in a cord T cell line, derived by cocultivating normal human cord leukocytes and human leukemic T cells. Nature 294, 770-771 (1981).

19. Miyoshi, I., Taguchi, H., Fujishita, M., Yoshimoto, S., Kubonishi, I., Ohtsuki, Y., Shiraishi, T. and Akagi, T., Transformation of monkey lymphocytes with adult T-cell leukemia virus. Lancer 1, 1016 (1982).

20. Miyoshi, I., Fijishita, M., Taguchi, H., Matsubayashi, K., Miwa, N. and Tanioka, Y., Natural infection in non-human primates with adult T-cell leukemia virus or a closely related agent. Int. J. Cancer 32, 333-336 (1983).

21. Miyoshi, I., Yoshimoto, S., Fujishita, M., Taguchi, H., Kubonishi, I. and Niiya, K., Natural adult T-cell leukaemia virus infection in Japanese monkeys. Lancet 18, 658 (1982).

22. Nalesnik, M.A. et al., The pathology of posttransplant lymphoproliferative disorder occuring in the setting of cyclosporine A-prednisone immunosuppression. Amer. J. Pathology 133 (1), 173-191 (1988).

23. Noda, Y., Ishikawa, K., Sasagawa, A., Honjo, S., Mori, S. and Tsujimoto, H., Hematological abnormalities similar to the preleukemic state of adult T-cell leukemia in African green monkey naturally infected with Simian T-cell leukemia virus. Jpn. J. Cancer Res., Dec. 77 (12), 1227-1234 (1986).

24. Ohtsuki, Y., Takahashi, K., Akagi, T., Ultrastructural comparison of human and monkey derived type C virus particles in monkey lymphoid cell lines, positive adult T-cell leukemia associated antigen (ATLA). J. Clin. Electron. Microscopy 18, 652 (1983).

25. Penn, I., Cancer following cyclosporine therapy. Transplantation 43, 32-35 (1987).

26. Sahai Srivastava, B.C., Wong-Staal, F. and Getchell, J.P., Human T-cell leukemia virus: provirus and antibodies in a captive gorilla with non-Hodgkin's lymphoma. Cancer Res. 46, 4756-4758 (1986).

27. Sakakibara, I., Sugimoto, Y., Sasagawa, A., Honjo, S., Nakamura, H. and Hayami, M., Spontaneous malignant lymphoma in an African green monkey naturally infected with Simian T-lymphotropic virus (STLV).

28. Starzl, T.E. et al., Reversibility of lymphomas and lymphoproliferative lesions developing under cyclosporine-steroid therapy. Lancet, March 17, 787 (1984).
29. Stevens HPJD, Hovius SER, Heeney JL, Nierop PWM, Jonker M; Immunological aspects and complications of composite tissue allografting for upper extremity reconstruction: a study in the rhesus monkey. Transpl Proc (in press), 1991; 23(1).
30. Stevens HPJD, Hovius SER, Vuzevski VD, Nierop PWM van, Gotte M, Roche N.A. Jonker, M; Immunological aspects of allogeneic partial hand transplantation in the rhesus monkey. Transpl Proc 22:2006, 1990.
31. Stevens HPJD, Kwast ThH van der, Meide PH van der, Vuzevski VD, Buurman WA, Jonker M. Synergistic immuinosuppressive effects of interferon gamma and tumor necrosis factor aplha. A skin transplantation study in the rhesus monkey. Transplantation 1990; 50(5)(in press).
32. Stevens HPJD, Roche N, Hovius SER, Jonker M: In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+, and MHC class II positive cells. Transpl Proc 22:1783, 1990.
32a. Swinnen LJ, Costanzo-Nordin MR, Fisher SG et al. Increased incidence of lymphoproliferative disorder after immunosuppression with the monoclonal antibody OKT3 in cardiac transplant recipients. N Eng J Med 323:1723-8 (1990).
33. Takatsuki K, Uchiyama T, Ueshima Y, Haltori T, Toibana T, Tsudo M, Wano Y, Yodi J: Adult T-cell leukemia: Proposal as a new disease and cytogenetic, phenotypic and functional studies of leukemic cells, Gann, Monograph on Cancer Research 28:13, 1982.
34. Touraine, J.L., Bosi, E., El Yafi, M.S., Chapuis-Cellier, C., Blanc, N., Dubernard, J.M., Piatti, P.M., Chevalier, M., Pouteil-Noble, C., Lenoir, G., Trepo, C., Gelet, A., Creyssel, R. and Traeger, R., The infectious lymphoproliferative syndrome in transplant patients under immunosuppressive treatment. Transplant. Proc. 17, 96-98 (1985).
35. Tsujimoto, A., Tsujimoto, H., Yanaihara, N., Abe, K., Hayani, M., Miwa, M. and Shimotohno, K., Detection of the X-gene product of Simian T-cell leukemia virus. FEBS-Letters, Febr. 17, 196 (2), 301-304 (1986).
36. Tsujomoto, H., Noda, Y., Ishikawa, K.I., Nakamura, H., Fukasawa, M., Sakakibara, I., Sasagawa, A., Honjo, S. and Hayami, M., Development of adult T-cell leukemia like disease in African green monkey associated with clonal integration of Simian T-cell leukemia virus type I. Cancer Res. 47, 269-274 (1987).
37. Watanabe, T., Seiki, M., Tsujimoto, H., Miyoshi, I., Hayami, M. and Yoshida, M., Sequence homology of the Simian retrovirus genome with human T-cell leukemia virus type 1. Virology 144, 59-65 (1985).
38. Watanabe, T., Seiri, M., Hirayama, Y., Yoshida, M., Human T-cell leukemia virus type 1 is a member of the African subtype of Simian viruses (STLV). Virology, Jan. 30, 148 (2), 385-388 (1986).
39. Yamamoto, N., Hinuma, Y., Zur Hausen, H., Schneider, J. and Hunsmann, G., African green monkeys are infected with adult T-cell leukemia virus or closely related agent. Lancet 1, 240-241 (1983).

.

CHAPTER IV: NEW APPROACHES TO OPTIMIZE IMMUNOSUPPRESSIVE THERAPY;

STUDY A : Synergistic immunosuppressive effects of monoclonal antibodies specific for interferon-gamma and tumor necrosis factor-alpha

H.P.J.D. Stevens

A.1 Introduction

In the complex network of cellular interactions of the immune response, cytokines play a crucial regulatory role. They are involved in the recruitment of immuno-competent cells into the site of inflammation and the amplification of the immune response, in which major histocompatibility complex (MHC) antigens play a key role.

Interferon gamma (IFN-γ) is known to exert a wide variety of immunomodulatory effects. It mediates the chemotactic migration and activation of T cells[1] and macrophages[2] and enhances the cytotoxic activity of natural killer cells, monocytes, cytotoxic T cells and polymorphonuclear leucocytes[3,4]. IFN-γ also enhances the expression of MHC class I and II antigens on almost every cell type, both in vitro[5,6] and in vivo[7,8] and it induces de novo synthesis of receptors for other cytokines such as tumor necrosis factor alpha (TNF-α)[9].

Recent evidence shows that TNF-α, which was originally found to induce hemorrhagic necrosis in murine tumors[10], also has widespread immunoregulatory activities. Like IFN-γ, TNF-α increases the expression of membrane glycoproteins such as ICAM-1, relevant for the adhesion of circulating granulocytes, monocytes and lymphocytes to endothelial cells[11,12]. TNF-α also exerts direct cytotoxic effects towards endothelial cells[13] and is involved in the regulation of both MHC class I and II antigen expression[14,15]. Because of these properties, it can be assumed that IFN-γ and TNF-α are both involved in the process of graft rejection.

To test this assumption the immunosuppressive effects of two monoclonal antibodies (MAbs) specific for human IFN-γ (MD1)[16] and human TNF-α (61E71)[15], were investigated using an allogeneic skin graft model in rhesus monkeys.

In vivo antibody-mediated interference with the function of lymphokines such as IFN-γ and TNF-a has been demonstrated to be effective in various studies. Recently, it was shown that anti-IFN-γ ameliorated experimental autoimmune diseases in rodents[17,18] and anti-TNF-a effected the pathophysiology of septic shock in mice and nonhuman primates[19,20]. Both MD1 and 61E71 were found to neutralize rhesus INF-γ and rhesus TNF-α but not murine IFN-γ or TNF-α. The rhesus monkey immune system, including the MHC gene products[21], show a high degree of similarity with the human counterparts. Therefore, results obtained in rhesus monkeys allow for better extrapolation to the human situation than data obtained from studies in rodents.

A.2   Materials and methods

A.2.1   Animals

Rhesus monkeys (Macaca mulatta) were born and raised in the TNO Primate Center, Rijswijk, employing a harem type breeding system. All monkeys were two to four years of age and typed for the rhesus leucocyte antigens (RhLA) -A, -B and -DR locus[21,22].

A.2.2   MAbs used for in vivo treatment

Monoclonal antibodies (MAbs) used for in vivo treatment were designated MD1, 61E71 and H105-1.6. MD1 (murine IgG1) was raised against recombinant human IFN-γ[16] and proved to fully neutralize the biological activity of naturally derived rhesus monkey IFN-γ. 61E71 (murine IgG$_1$) was raised against recombinant human TNF-α[15] and also proved to effectively neutralize rhesus monkey TNF-α. Neither of these MAbs exerted a neutralizing effect towards IFN-γ and TNF-α of rat or mouse origin. H105-1.6 is a murine anti-human MAb (IgG$_{2a}$) specific for human secretory component and not reactive with rhesus secretory component[23] 61E71 was produced in vitro (generously donated by Celltech, Ltd. Slough, Berkshire, UK). All other antibodies were purified from ascites by ammonium sulphate precipitation (50%) saturation. MD1 and 61E71 were also purified by adsorption to and elution from protein A. Prior to in vivo administration all MAbs were centrifuged for 20 minutes at 100.000 x g and sterilized by millipore filtration (0,22μM).

A.2.3   Skin grafting

Skin grafts were exchanged between non-immunized unrelated monkeys as described elsewhere[24]. Major hemorrhages and crust formation over the entire graft was taken as the endpoint of graft survival. Donor and recipients differed for all MHC antigens, both class I (RhlA-A & B) and class II (RhLA-DR).

A.2.4 Experimental groups

The skin graft experiment concerned five different experimental groups. In group I, animals did not receive a treatment. Five of these monkeys were historic controls[25,a]. In group II animals received H105-1.6. In this group skin graft recipients differed for one RhLA-A or -B antigen only. Results from group II have been published previously[23]. In groups III and IV animals received either MD1 or 61E71. Animals in group V were treated with the combination of MD1 and 61E71. Based on previous experiments, doses were used that would establish a small excess in serum levels of each injected antibody. Recipients received a daily doses of 1.3 mg/kg of MD1, 2.0 mg/kg of 61E71 or 1.0 mg/kg of H105-106 as an i.v. bolus injection for the duration of 10 days starting day -1, one day before skin transplantation.

A.2.5 Histological Studies

At regular intervals after operation, biopsies were taken from the skin grafts of two animals in group I and all animals in groups IV and V. After excision with a 3 mm^2 biopsy stance a cylinder of full thickness skin was cut in half, longitudinally. One half was fixed in buffered formalin and processed routinely for histology on hematoxylin and azofloxine-stained sections.

The remaining other half, snapfrozen in liquid nitrogen chilled isopentane, was used for parallel immunohistochemical studies as described elsewhere[26]. Tissue bound primary antibody was reacted with horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS containing 5% heat-inactivated normal rhesus serum and 5% normal rabbit serum. Conjugate binding was visualized with a solution of 0.5 mg/ml 3,3,-diaminobenzidine tetrahydrochloride (Fluka Chemika, FRG) and 0.03% H_2O_2 in PBS. The following antibodies were used for immunohistological staining: W6/32[27] (Serotec, Blackthorn, GB) specific for MHC class I antigens; anti-HLA-DR specific for MHC class II-DR antigens[28] (Becton and

---

[a]Previous data have shown that in completely RhLA-A, -B and -DR mismatched donor-recipient combinations, mean skin graft survival time is 9.1 days (SE= 0.4 days, n=5) [25], while the day of operation was counted as day 1. However, in this study we prefer to regard the day of operation as day zero in accordance to international standards.

Dickinson, Mountainview, USA); the MAb T11[28] (DAKO-T11, Dakopatts, Denmark) specific for CD2, as a pan T cell marker; a mixture of OKT4 and OKT4A[30] (Ortho Pharmaceuticals Co., Raritan, NJ) specific for CD4+ cells and GM9[31] specific for CD8+ cells. All MAbs are fully reactive with rhesus monkey antigens. In control incubations the primary antibody was omitted.

A.2.6 Hematology and Subset Analysis

Blood samples were taken at regular time intervals and the total number of leucocytes as well as the relative number of lymphocytes, monocytes and granulocytes were determined in all animals. In groups II to V relative and absolute numbers of peripherally circulating mature T cells, CD4+, CD8+ and class II+ cells were determined, using an indirect immunofluorescence technique as described elsewhere[32].

A.2.7 Determination of level of injected MAb

In all groups serum levels of injected MAb were determined in a sandwich enzyme-linked immunosorbent assay (ELISA). The first layer was affinity purified goat anti-mouse IgG (Pel-Freeze Biologicals, Rogers, AR) coated on flexible 96-well assay plates (Falcon 3911, Oxnard, CA). The intermediate layer was serum diluted 1/10 or MD1 and 61E71 standards in a similar serum dilution. Binding was detected with affinity purified and peroxidase labelled goat anti-mouse IgG (Pel-Freeze). Color development was initiated with O-phenylenediamine (Kodak, Rochester, NY) in 0.03% H_2O_2 PBS and terminated with 2N H_2SO_4. The plates were read at 492 nm. (Titertek Multiskan PLUS MKII, Mc Lean, Virginia). For quantitation of the amount of circulating MAb calibration curves were used of MD1 and 61E71 of a known concentration.

A.2.8 Determination of Monkey-anti-mouse IgG antibodies

The presence of anti-mouse IgG antibodies was determined in serum samples by a similar ELISA technique[32]. In this assay, the last ten-fold dilution that still showed a significant higher level (>2 times SD) of anti-mouse IgG antibodies to MD1 or 61E71 than the control, was taken as the titer of the sample.

A.2.9 Assays for the determination of IFN-γ and TNF-α

In groups II to V, serum IFN-γ levels were assessed semi-quantitatively in a cytopathic inhibition assay in HEp-2 cells with Vesicular Stomatitus Virus (VSV) as challenge[16]. Cell death was monitored with 0.5% crystal-violet staining.

The presence of biologically active TNF-α in serum was determined in a bioassay by measuring cell death of a murine WEHI 164 clone 13 fibrosarcoma cell line[33] briefly WEHI 164 cells were seeded at a density of 4×10^4 cells per well in 96-well microtiter plate in 100 μl RPMI, containing 10% FCS. An equal volume of each serum sample was added in serial two-fold dilutions and incubated at 37° C for 24 hours in a humidified CO_2 incubator. Subsequently cells death was assessed with the tetrazolium salt method[34].

A.3   Results
A.3.1 Effects on skin allograft survival

Untreated control animals had a mean skin graft survival time (MST) of 8.3 +/- 0.7 days (Table 1). MST were not significantly prolonged by the administration of the irrelevant MAb H105-1.6[23] nor by MD1. The MST values in these groups were 8.2 and 9.5 days respectively. In the group of animals treated with only 61E71, a slight, non-significant prolongation of skin graft survival was observed (MST = 10 days) as compared with the untreated control group. However, prophylactic treatment with the combination of MD1 and 61E71 (group V) resulted in a modest, but significant prolongation of graft survival times to a MST of 12.3 days (Mann-Whitney U test $p < 0.05$).

A.3.2 General histology

Histology was performed on skin grafts of two monkeys in group I and all animals in groups IV and V. All allografts investigated showed a similar sequence of histopathological events, although at different rates. Infiltrates of lymphocytes and histiocytes, capillary destruction and hemorraghe started perivascularly. Subsequent microscopical changes reflected macroscopical findings of the proces of skin graft rejection.

Table 1. Skin allograft survival times in rhesus monkeys treated with MAbs
specific for IFN-γ and TNF-α.

Experimental groups	Injected MAbs[a]	Target	Individual skin graft survival times (days)
I	none	-	7, 7.5[b], 8[b], 8[b], 8, 8, 9[b], 9[b], 9, 9
II	H105-1.6[c]	-	8, 8, 8.5
III	MD1	IFN-γ	9, 9
IV	61E71	TNF-α	10, 10
V	MD1 & 61E71	IFN-γ & TNF-α	10.5[d], 12, 13, 13.5

a) Monoclonal antibody (MAb) treatment was started 1 day before skin
    grafting and continued for 10 days.
b) Historic controls
c) H105-1.6 is an irrelevant murine IgG2a monoclonal antibody[23].
d) rhesus monkey 1ZV.

Lymphohistiocytic infiltrates and edema became more diffuse and finally extensive hemorraghe, epidermiolysis and crust formation developed as rejection of the skin graft was definite.

A.3.3 Immunohistology

Immunohistology was confirmative. The intensity of MHC class I and II-DR antigen expression by the stromal cells of the papillar and reticulary dermis, vascular endothelium and epidermal cells increased when rejection progressed (Table 2). If infiltrates were only focal, MHC class II-DR expression was largely confined to the area around the infiltrating cells. At 9 days after transplantation all preexisting structures and infiltrates in the allografts of group I and IV showed very intense expression of MHC class I and II-DR antigens (Fig. 1a and 1c).

Table 2. Immunohistological observations in rhesus monkey skin grafts after various treatments.

Group(n)		Therapy	Days postop.	Expression on preexisting donor tissue of		Mean percentage of infiltrate with expression of		
				MHCclassI	MHCclassII	CD2	CD4	CD8
I	(2)	-	5	+ + +	+ + (focal)	20	10	10
			7	+ + +	+ + + (focal)	60	< 5	60
			9	+ +/+ + +	+ +/+ + +	80	< 5	80
IV	(2)	61E71	5	+	+/-	nd	nd	nd
			7	----------------------- not tested -------------------------------				
			9	+ + +	+ +/+ + +	85	< 5	85
			13	+ +	+ +	85	< 5	85
V	(3)	61E71	5	+/-	-	nd	nd	nd
		& MD1	7	+	-	30	< 5	30
			9	+ +	+ +	65	< 5	65
			13	+/+ +	+/+ +	50	< 5	50
V*	(1ZV)	61E71	5	+	+/-	nd	nd	nd
		& MD1	7	+ +	+ +	70	< 5	70
			9	+ + +	+ + +	50	< 5	50

Data represent consistent observations made in all skin grafts of the experimental groups I (n=2), IV (n=2) and V except for monkey 1ZV (n=3). Results for monkey 1ZV are given separately under V*. MAbs used for staining lymphocytes are described in section 2.7. Staining of the intensity of MHC class I and II antigen expression on the stroma of the papillary and reticular dermis and on epidermal cells was scored semiquantitatively: + for low, + + for moderate, + + + for high intensity of antigen expression. nd = not determined as the number of infiltrated cells was too small to determine percentages.

At the same time in group V, except for monkey 1ZV, allografts showed a less prominent expression of MHC class I and II-DR antigens (Fig. 1b and 1d).

At 7 days after operation, in all allografts, less than 5% of the CD2+ cells were positive for OKT4 and 4A, implying that only very few CD4+ cells were present at the time of fullblown rejection. Almost all T cells were of the CD8+ phenotype. This CD4/CD8 ratio sustained throughout the rest of the rejection process.
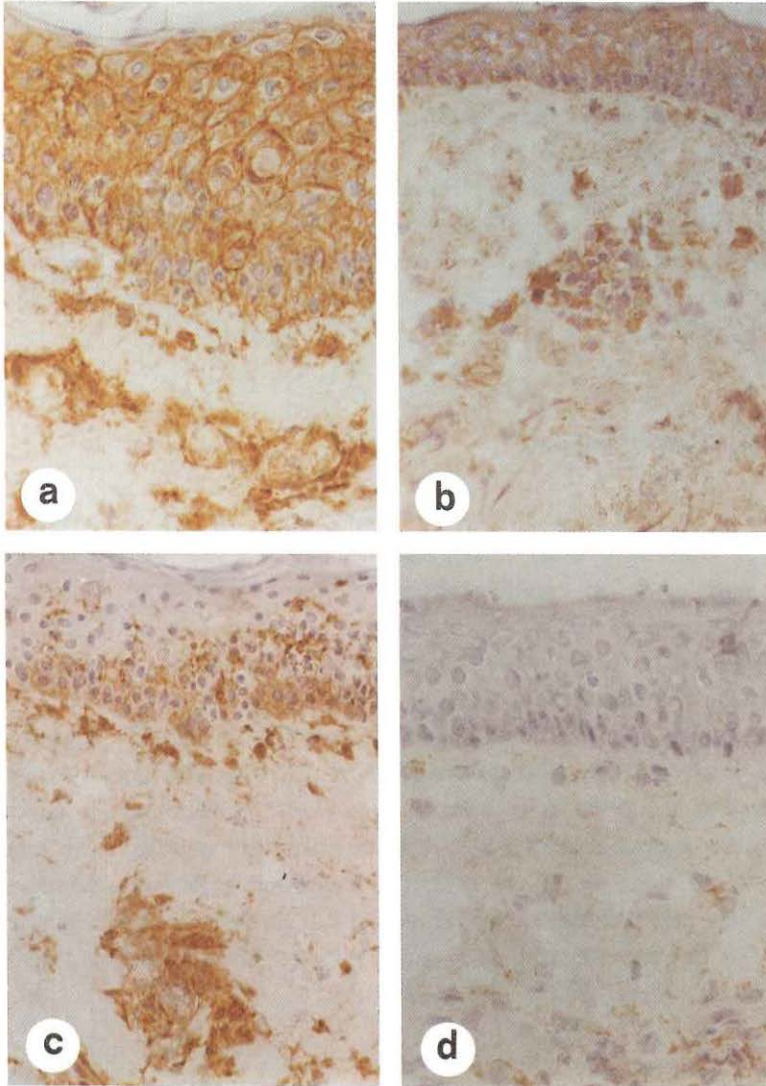
Figure 1. Immunohistological staining of skin biopsies with MAbs specific for MHC class I (fig 1a, 1b) and MHC class II (fig 1c, 1d) antigens. All biopsies were taken 9 days after skin grafting. At this time, intense expression of MHC class I and II antigens could be seen on epidermis, dermis and infiltrates of the allografts of the monkeys that had received 61E71 (fig. 1a, 1c). At the same time, MHC class I and II antigen expression on epidermis, dermis and infiltrates of the skin biopsies of the monkeys that had received the combination of MD1 and 61E71 was less prominent (fig. 1b, 1d; magnification 275x).

A.3.4 Effect on hematology and lymphocyte subsets

Lymphopenia did not occur, nor was there a depression in platelet, leucocyte, granulocyte, or monocyte counts during or after the 10-day course of intravenous therapy in any of the animals.

The fluctuations in absolute or relative numbers of CD3+, CD4+, CD8+ and MHC class II-DR+ PBLs did not significantly differ from preoperative levels and thus no correlations were found between subset fluctuations in peripheral blood and clinical events such as skin transplantation, administration of MAbs or rejection of the allograft (data not shown).

A.3.5 Serum levels of antibody

In all cases, the daily injected doses of MAbs were sufficient to maintain detectable serum trough levels until about 1 day after the last injection. Trough levels, assessed by ELISA, varied from 35 ng/ml to 380 μg/ml during 9 days after transplantation. On day 12 postoperatively, levels were all below detection level. Although there was significant variation in levels among the individual monkeys, no significant differences in MAb levels were observed between the different groups.

Antibody formation in the rhesus monkey directed to the injected murine MAb was also determined by ELISA. None of the monkeys had detectable levels of monkey-anti-mouse antibodies before the start of experiments (Table 3). However, in all cases these antibodies were detectable from the 9th postoperative day onward. Peak titers ranged from 1 in 10^3 to 1 in 10^6. In one case, anti-mouse-antibodies were already detectable on the 7th day after operation against both of the MAbs that were injected. This monkey (1ZV) had received both MD1 and 61E71 (group V) and had a relative short skin graft survival time of 10.5 days (Table 1).

A.3.7 Serum levels of IFN-γ and TNF-α

Only in an incidental case the presence of IFN-γ and TNF-α could be established in serum, preoperatively. Peri- and postoperative fluctuations in serum levels did not correlate with any of the afore mentioned clinicopathological events (data not shown).

Table 3.   Monkey-anti-mouse antibody titers[a] in rhesus monkeys treated with
MAbs against IFN-γ and TNF-α to prevent skin allograft rejection.

group	monkey	treatment	target of reactivity[b]	d-5	d7	d9	d14	d28
III	1NS	MD1	MD1	0	0	3	5	5
	2V	MD1	MD1	0	0	3	4	4
IV	1NG	61E71	61E71	0	0	0	6	
	B8	61E71	61E71	0	0	0	5	5
V	1YV	combi	MD1	0	0	3	5	5
			61E71	0	0	0	3	3
	BB36	combi	MD1	0	2	2	6	5
			61E71	0	0	3	4	4
	1ZV	combi	MD1	0	2	2	4	4
			61E71	0	2	2	4	5
	2BK	combi	MD1	0	0	0	2	4
			61E71	0	0	0	3	4

[a]The last ten-fold dilution that still showed a significant higher level (>2 times SD) of
anti-mouse IgG antibodies to MD1 or 16E71 than the control, was taken as the titer of the
sample.
[b]Levels of monkey-anti-mouse antibody titers were assessed by ELISA to both MD1 and 61E71
in all samples.

A.4   Discussion

The role of IFN-γ and TNF-α in the process of allograft rejection was investigated
in a rhesus monkey model. Two murine MAbs, MD1[16] and 61E71[15] specific for IFN-
γ and TNF-α respectively, were tested for immuno-suppressive activity in a skin
transplantation study. Prophylactic treatment of allogeneic skin graft recipients with MD1
(group III) or 61E71 (group IV) alone did not prolong skin graft survival times.

Neutralization of just either one of these cytokines, was, apparently, not sufficient to suppress the immune response. However, treatment with the combination of MD1 and 61E71 (group V) resulted in a modest but significant prolongation of allograft survival times. Compared to MAbs specific for MHC class II positive and CD8+ cells, which had MST of 12.4 and 12.9 days[35,36,a] respectively, the immunosuppressive effect of the combination of MD1 and 61E71 was equally strong. CD3 and CD4 specific MAbs and the combination of MAbs specific for CD3, CD4, CD8 and MHC class II antigens proved to be more effective and could prolong MST from 14.6 up to 19.3 days[23,32,35,a]. The fact that the immunosuppressive effect of anti-IFN-γ and anti-TNF-α was only temporary, might result from the presence of other lymphokines than IFN-γ and TNF-α that additively take over the upregulation of the immune response[37]. Grid histology revealed that, except in the case of monkey 1ZV, all grafts in group V were infiltrated two to four days later by lymphocytes and histiocytes than the grafts in group IV. The evolution of the rejection process, however, was similar in all cases, although retarded in the allografts of group V. Immunohistology was confirmative. Furthermore, from the observations as summarized in Table 2, we conclude that at the time of full blown rejection of an allogeneic skin graft in the rhesus monkey, almost all T cells are of the CD8+ phenotype. MD1 nor 61E71, alone or in combination, influenced this phenomenon. Striking was that MD1 in combination with 61E71 delayed the recruitment of lymphocytes and macrophages into the graft. Secondly, the onset of MHC class I and II antigen expression was delayed on preexisting donor tissue in group V compared to group I and IV.

In the peripheral blood, however, no effect in any of the groups could be found on lymphocyte subset distributions. This might be due to the fact that the injected MAbs were primarily directed against local acting lymphokines. On the other hand, the size of the grafts were most likely too small to influence subset fluctuations in such a manner that they would reflect the local immunological events of the graft. Also the serum levels of IFN-γ and TNF-α did not correlate to clinico-pathological events. These results suggest that serum levels of IFN-γ and TNF-α do not reflect the presence of these lymphokines on the site of allograft rejection.

Neither can the augmented immunosuppressive effect of the combination of MD1 and 61E71 be explained on basis of longer bioavailability of these MAbs in circulation compared to the groups where the MAbs were given separately. For, in all cases, the daily injected doses of MAbs were sufficient to maintain detectable serum levels until approximately 1 day after the last injection. Furthermore, in all treated skin graft recipients, similar titers of anti-mouse-antibodies could be detected from 9 days after

operation onwards.

A possible explanation of the effectiveness of MD1 and 61E71 might be related to interference with the mediatory role of IFN-γ and TNF-α on a combination of several different pathways. Firstly, the recruitment of lymphohistiocytic cells into the allograft was delayed. MD1 and 61E71 MAb might have interfered with the induction of expression of endothelial adherence molecules such as ICAM-1, ELAM-1[11] and ENA1[12] and the migration stimulus for lymphocytes and macrophages to cross vascular endothelium[1,2].

Secondly, the combination of MD1 and 61E71 impaired the upregulation of MHC class I and II antigen expression (Table 2). As a result antigenicity of the grafts might be reduced[38-40], and enhancement of antigen presentation to T helper lymphocytes and the subsequent induction of cell-mediated cytoxicity[41] might be impaired.

Thirdly, TNF and IFN-γ influence each others production. TNF is primarily produced by activated monocytes and macrophages[42,43] but also by T lymphocytes and Natural Killer cells[44]. The production of TNF by these cells, however, is dependent on the presence of proliferating IFN-γ producing T cells[45]. By intervening in these interactions anti-IFN-γ and anti-TNF-α MAbs might also have prevented the cascade of cytokine production and thus preventing amplification of the immune response.

In conclusion, for the first time in an in vivo transplantation model, a synergistic effect of anti-IFN-γ MAb (MD1) and anti-TNF-α MAb (61E71) is described, resulting in a delayed onset of the immune response towards an allogeneic skin graft. The described synergism might be due to interference with the effects of IFN-γ and TNF-α on several separate pathways whose combined action is greater than the sum of their separate actions. To gain a better insight in this proces, similar experiments should be performed in which administration of the MAb therapy specific for combinations of different cytokines is started at different times before and after transplantation.

A.5   References

1.   Issekutz TB, Stolz JM, Meide PH van der. Lymphocyte recruitment in delayed-type hypersensitivity: the role of IFN-γ. J Immunol 1988; 140: 2989.
2.   Nathan CF, Kaplan G, Levis WR, et al. Local and systemic effects of intradermal recombinant interferon-γ in patients with lepromatous leprosy. N Engl J Med 1986; 315: 6.
3.   Herberman RB. Ch. Interferon & Cytotoxic Effector Cells in: Vilceck & DeMaeyer (Eds.) Interferon & the Immune System. Elsevier, Amsterdam 1984; 61.
4.   Trinchieri G, Matsumoto-Kobayashi M, Clark SV, Sheera J, London L, Perussia B. Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 1984; 160: 1147.

5. Fellous M, Nir U, Wallach D, Merlin G, Rubinstein M, Revel M. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. PNAS 1982; 79: 3082.
6. Wong GHW, Clark-Lewis I, Harris AW, Schrader JW. Effect of cloned interferon-γ on expression of H-2 and Ia antigens on cell lines of hemopoietic, lymphoid, epithelial, fibroblastic and neural origin. Eur J Immunol 1984; 14: 52.
7. Momburg F, Koch N, Moller P, Moldenhauer G, Hammerling GJ. In vivo induction of H-2K/D antigens by recombinant interferon-γ. Eur J Immunol 1986; 16: 551.
8. Steiniger B, Meide PH van der, Westermann J, Klempnauer J. Systemic induction of class II MHC antigens after continuous intravenous infusion of recombinant gamma interferon in rats. Transpl Proc 1987; XIX (5): 4322.
9. Ruggiero V, Travenier J, Fiers W, Baglioni C. Induction of the synthesis of tumor necrosis factor receptors by interferon-γ. J Immunol 1986; 136 (7): 2445.
10. Carswell EA, Old LJ, Kassel RL, Green S, Fiore NC, Williamson BD. An endotoxin-induced serum factor that causes necrosis of tumor. PNAS 1975; 72: 3666.
11. Pober JS, Gimbrone MA, Lapierre LA, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. J Imunnol 1986; 137: 1893.
12. Leeuwenberg JFM, Jeunhomme TMAA, Buurman WA. Induction of an activation antigen on human endothelial cells in vitro. Eur J Immunol 1989; 19: 715.
13. Sato N, Goto T, Haranaka K. et al. Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition and cytotoxicity. JNCI 1986; 76: 1113.
14. Collins T, Lapierre LA, Fiers W, Strominger JL, Pober JS. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. PNAS 1985; 83: 446.
15. Leeuwenberg JFM, Damme J van, Meager T, Jeunhomme TMAA, Buurman WA. Effects of tumor necrosis factor on the interferon-γ-induced histocompatibility complex class II antigen expression by human endothelial cells. Eur J Immunol 1988; 18: 1469.
16. Meide PH van der, Dubbeld M, Schellekens H. Monoclonal antibodies to human immune interferon and their use in a sensitive solid-phase ELISA. J Immunol Meth 1985; 79: 293.
17. Jacob CO, Meide PH van der, McDevitt HO. In vivo treatment of (NZBxNZW)F₁ lupus-like nephritis with monoclonal antibody to gamma interferon. J Exp Med 1987; 166: 798.
18. Jacob CO, Holoshitz J, Meide PH van der, Strober S, McDevitt HO. Heterogeneous effects of IFN-γ in adjuvant arthritis. J Immunol 1989; 142: 1500.
19. Beutler B, Milsark IW, Cerami AC. Passive immunnization against Cachectin/Tumor Necrosis factor protects mice from lethal effect of endotoxin. Science 1985; 229: 869.
20. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 1987; 330: 662.
21. Vreeswijk W van, Roger JH, D'Amaro J, Balner H. The major histocompatibility complex of the rhesus monkey, RhL-A; VII. Identification of five new serologically defined antigens. Tissue Antigens 1977; 9: 17.
22. Roger JH, van Vreeswijk W, Balner H. The major histocompatibility complex of rhesus monkeys; XIII. Current knowledge of DR and other B-cell specific antigens. J Immunogen 1980; 7: 333.
23. Nooij FJM, Jonker M. The effect on skin allograft survival of a monoclonal specific for a

polymorphic CD-3 like cell surface molecule in rhesus monkeys. Eur J Immunol 1987; 17: 1089.

24. Balner H. Skin grafting in monkeys and apes. Transplantation 1969; 8: 206.

25. Vreeswijk W, Balner H. Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 1980; 30 (3): 196.

26. Chadha S, Kwast ThH van der, Immunohistochemistry of ovarian granulosa cell tumours; the value of tissue specific proteins and tumour markers. Virchows Archiv A Pathol Anat 1989; 414: 439.

27. Parham P, Barnstable CJ, Bodner WF. Use of monoclonal antibody (W6/32) in structural studies of HLA-A,B,C antigens. J Immunol 1979; 123: 342.

28. Banks PM, Caron L, Morgan TW. Application of monoclonal hybridoma antibodies to imprint preparations of lymphoid tissue for the identification of immunological cell types.
Am J Clin Path 1982; 78: 266.

29. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J Immunol 1981; 126: 2117.

30. Jonker M, Neuhaus P, Zurcher C, Fucello A, Goldstein G. OKT4 and OKT4A antibody treatment as immunosuppression for kidney transplantation in rhesus monkeys. Transplantation 1985; 39: 247.

31. Jonker M, Van Meurs GJE. in Bernhard A, Boumsell L, Dausset J, Milstein C, Schlossman SF.(Eds.). Leucocyte Typing, Springer-Verlag, Berlin 1984; 8: 206.

32. Jonker M, Goldstein G, Balner H. Effects of in vivo administration of monoclonal antibodies specific for human T cell subpopulations on the immune system in a rhesus monkey model. Transplantation 1983; 35(6): 521.

33. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 1986; 95: 99.

34. Hansen MB, Nieksen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989; 119: 203.

35. Stevens HPJD, Roche N, Hovius SER, Jonker M. In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive cells. Transpl Proc 1990; (in press).

36. Jonker M, Nooij FJM, Suylichem P van, Neuhaus P, Goldstein G. The influence of OKT8F treatment on allograft survival in rhesus monkeys. Transplantation 1986; 41(4): 431.

37. Groenwegen G, Ley M de, Jeunhomme GMAA, Buurman WA. Supernatants of human leukocytes contain mediator, different from interferon-γ, which induces expression of MHC class II antigens. J Exp Med 1986; 164: 131.

38. Milton AD, Fabre JW. Massive induction of donor-type class I and class II major histocompatibility complex antigens in rejecting cardiac allografts in the rat. J Exp Med 1985; 61: 98.

39. Havry P, Willebrand E von. The influence of the pattern of inflammation and administration of steroids on class II MHC antigen expression in renal transplants. Transplantation 1986; 42 (4): 358.

40. Ferry B, Halttunen J, Leszczynski D, Schellekens H, Meide PH van der, Havry P. Impact of class II major histocompatibility complex antigen expression on the immunogenic potential of isolated rat vascular endothelial cells. Transplantation 1987; 44 (4): 499.

1. Engleman EG, Benike JL, Grumet FC, Evans L. Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. J Immunol 1981; 127 (5): 2124.
2. Beutler B, Cerami A. Cachectin: more than a tumor necrosis factor. N Eng J Med 1987; 316: 379.
3. Beutler B, Krochin N, Misark IW, Luedke C, Cerami A. control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science 1986; 232: 977.
4. Cuturi MC, Murphy M, Costa Giomi MP, Weinmann R, Perussia B, Trichinieri G. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med 1987; 165: 1581.
5. Debets JMH, Linden CJM van der, Spronken IEM, Buurman WA.
    T cell-mediated production of tumour necrosis factor-α by monocytes. Scand J Immunol 1988; 27: 601.

CHAPTER IV: NEW APPROACHES TO OPTIMIZE IMMUNOSUPPRESSIVE THERAPY;

STUDY B   : Combined effects of low-dose FK-506 and CyA on skin graft survival

H.P.J.D. Stevens & S.E.R. Hovius

B.1 Introduction

FK-506 is a newly developed drug with impressive immunosuppressive properties. There is reluctance to use the drug clinically because of the reported toxicity of FK-506[1]. However, recent results obtained in subhuman primates permit some optimism[2]. It is likely that the future of FK-506 will lie in its cautious use in combination with other immunosuppressive agents.

An obvious candidate to be combined with FK-506 is cyclosporine (CyA), the more so as there are indications that both drugs act synergistically[3,4]. The present experiments were performed to investigate the possible synergism and toxicity of low-dose FK-506 and low-dose CyA using a rat skin transplantation model.

B.2 Materials and methods

B.2.1 Animals

Male rats of the Rt-1 incompatible BN and WAG strains were used respectively as donors and recipients. The animals were bred under specific pathogen free conditions and weighed about 250 gr.

B.2.3 Skin transplantation

Full thickness skin grafts were transplanted to the dorsal thorax according to the method described by Balner[5]. The compression bandages were removed after 8 days after which the grafts were inspected daily. The day on which the graft had necrotized more than 90 % was taken as day of rejection.

B.2.4 Immunosuppression

FK-506, kindly supplied by the Fujisawa Pharmaceutical Co., Osaka, Japan, was dissolved in olive oil and administered intramuscularly (i.m.) in dosages of 0.1, 0.3 and 0.5 mg/kg per day. CyA (Sandoz), obtained from a commercial batch, was diluted in olive oil and given i.m. at a dose of 5 mg/kg/day. Both drugs were given daily, either alone or combined, for 15 days, starting on the day of transplantation. Each group consisted of 5 animals.

B.2.5 Toxicity studies

Blood samples were taken before and at regular intervals after grafting for blood cell countings and determination of glucose, haemoglobin (Hb), SGOT and creatinine levels. The animals were weighed regularly.

B.3 Results and discussion

The survival times obtained in the different experimental groups are summarized in table I. Untreated controls had a mean graft survival time (MST) of 9.6 days. Treatment with CyA alone, at a dose of 5mg/kg/day, resulted in a significant prolongation of graft survival (MST of 18.0 days). Animals treated with 0.1, 0.3 or 0.5 mg/kg FK-506 showed a dose-related prolongation of skin graft survival. The MST's were significantly different from the controls and from each other; they amounted to 16.2, 21.6 and 25.8 days, respectively. Administration of 0.1 mg FK-506 had a similar effect on graft survival as 5 mg CyA. Considering the fact that a skin graft represents a difficult tissue barrier as compared with a heart or kidney graft, the survival times obtained with the higher dosages of FK-506 were remarkable.

However, using these dosages a mild toxicity, manifested by a slight and reversable increase in SGOT levels, was noted.

Other parameters remained normal. The combined treatment with CyA and and 0.1 mg FK-506 led to a MST of 28.2 days; which is significantly longer than the MST of any other group, except for the group treated with 0.5 mg FK-506. The prolongation in graft survival time obtained in the combined treatment group was longer than the sum of prolongations observed in the component groups. In the combination group toxicity studies revealed no drug related changes in body weight, blood counts, Hb, glucose , SGOT or creatinine levels.

The present results are in agreement with earlier studies, indicating that minimally therapeutic doses of FK-506 act synergistically with suboptimal doses of CyA[3,4]. In a dog kidney transplantation model it has been demonstrated that this synergism could be best exploited if prednisone was added to the immunosuppressive cocktail[6]. Synergism in immunosuppressive activity with FK-506 combined with irradiation in hamster to rat xenografting has also been described[7].

Table 1. Effect of FK-506 and CyA on skin allograft survival in rats

Treatment	Dose	Survival time in days	Mean +/- SD
Controls		9, 9, 10, 10, 10	9.6 + 0.5
CyA	5 mg/kg	17, 18, 18, 18, 19	18 +/- 0.7
FK-506	0.1 mg/kg	15, 16, 16, 17, 17	16.2 +/- 0.8
FK-506	0.3 mg/kg	20, 21, 21, 23, 23	21.6 +/- 1.3
FK-506	0.5 mg/kg	24, 25, 26, 27, 27	25.8 +/- 1.3
FK + CyA	0.1 + 5 mg/kg	26, 28, 28, 29, 30	28.2 +/- 1.5

Skin transplantation was performed in the WAG to BN rat combination. CyA and Fk-506 were given im. daily for 15 days starting on the day of transplantation. The combined treatment group is significantly different from all other groups except the group treated with 0.5 mg FK-506. The results obtained with 0.1, 0.3 and 0.5 mg FK-506 are significantly different from each other and the controls ($P < 0.05$).

These data collectively emphasize the marked potentials of low-dose FK-506 as part of triple or quadruple therapy. Especially with regard to difficult organs like the pancreas and the small bowel or with regard to composite grafts, FK-506 may become a valuable adjunct to the existing immunosuppressive arsenal.

It may also be useful in highly immunized patients and in xenografting. The redemption of these prospects depend on the outcome of the first clinical trials which are currently being performed.

B.4 References

1. Collier DS, Thiru S, Calne R: Transplant Proc 19:62, 1987.
2. Todo S, Demetris A, Ueda Y et al.: Surgery 106:444, 1989.
3. Murase N, Todo S, Lee PH et al. Transplant Proc 19:71,1987
4. Zeevi A, Duquesnoy R, Eiras G et al.: Transplant Proc 20:220, 1988
5. Balner H Transplantation 2:464, 1964
6. Todo S, Demetris A, Ueda Y et al. Transplant Proc 19:57, 1987
7. Ochiai T, Nagata M, Nakajiama K et al. Transplant Proc 19:84, 1987

CHAPTER V: DISCUSSION & SUMMARY

S.E.R. Hovius & H.P.J.D. Stevens

This 'double-thesis' provides data about the technical, functional and immunological aspects of transplantation of the allogeneic (radial side of the) hand investigated in a rhesus monkey model.

Research in this field was prompted by the question from patients and/or parents as to whether the problem of a nonfunctioning or absent hand could be solved by allogeneic transplantation of a hand. Whatever the original cause; traumatic, congenital or neoplastic, even the most sophisticated prothesis can not restore the lost motor and sensory functions of the original hand.

Nowadays, almost any organ can be transplanted clinically, varying from the kidney[1,2], heart (sometimes in combination with lung)[3], liver[4], bone marrow[5], pancreas[6], intestines[7] and skin[8]. Especially with the advent of Cyclosporine A (CyA) (Chapter I, section B.3.1.4: ChI.B.3.1.4) reasonable to very good graft and patient survival rates can be obtained[1-7]. Moreover, since the sixties, microsurgical techniques have become available that make it possible to suture very small vessels and nerves (ChI.A.1). Because of these advancements we have investigated in an experimental model whether transplantation could be added to the arsenal of modern treatments for hand reconstruction.

As mentioned previously (ChI), multiple studies have been performed focussing on the feasibility of composite tissue allograft (CTA) transplantation under various kinds of immunosuppression, but mainly in a rat limb transplantation model[9-17]. Rodent models for tissue transplantation give leads as to what technical, functional and immunological aspects are of importance. In this respect, it could be demonstrated by our group that fluctuations in peripheral blood gases as well as glucose and lactate did not predict allograft rejection of the CTA (ChII.A). Furthermore, the flux of peripheral blood cells through the skin of the CTA, as could be assessed by laser doppler flowmetry, decreased concomitantly with histological and clinical parameters indicating increment of graft rejection. However, these laser doppler flow measurements could not predict the initial onset of rejection, before the occurrence of clinical signs such as edema, loss of hair and epidermolysis. Presumably, this was due to the lack of continuous monitoring using this method.

For optimal extrapolation to the situation in man, studies in rodents have clear limitations from a technical, a functional as well as an immunological point of view. Should transplantation of the (total) hand in man ever be performed, then information on these three topics will be of ultimate importance.

Preferably a nonhuman primate should be used for preclinical testing of these aspects of CTA-transplantation, because of the obvious anatomical, functional, immunological and phylogenetic similarities between monkey and man.

Also for these reasons nonhuman primates are known to fulfill the requirements for testing the efficacy and safety of immunosuppressive drugs (such as monoclonal antibodies)[18,19]. It was therefore our objective to study the afore mentioned aspects of allogeneic hand transplantation in a rhesus monkey model. In the experimental model the transplant consisted of the radial side of the hand enlarged with the radial forearm flap (Fig.1, ChIC.1.2).For ethical reasons a small independent functional unit of the hand was chosen. One of the introductory studies indeed proved that the anatomy of the transplant of the rhesus monkey was practically identical to the situation in man (ChII,B). The rhesus monkey lives on the ground as well as up in the trees. Because of this pattern of life the rhesus monkey needs a better functioning thumb in comparison to arboreal monkeys. The rhesus monkey can bring its fingers into opposition, however not as well as in the

Figure 1. The transplant model. The dotted line marks the radial hand unit. Ventral: A = adductor muscle; T = thenar muscle; U.n. = ulnar nerve; M.n. = median nerve; f.p.l. = flexor pollicis longus; a.p.l. = abductor pollicis longus; r.a. = radial artery. Dorsal: e.p.l. = extensor pollicis longus; a.p.l. = abductor pollicis longus; s.r.n. = superficial radial nerve; c.v. = cephalic vein.

human being. The thumb of a rhesus monkey is shorter in comparison with that in man. Important differences were the fact that the long flexor muscle (musculus flexor pollicis longus) to the thumb only separated from the other flexor muscles at the level of the wrist. Because of this, flexion of the thumb is less independent. Also, the monkey misses the short extensor muscle (musculus extensor pollicis brevis) resulting in less stability of the metacarpometaphalangeal joint of the thumb during grasp movements. An important resemblance between rhesus monkey and man was the fact that the thenar muscles appeared to be built up in a similar way. Also the nerves to the thumb followed an almost identical course.
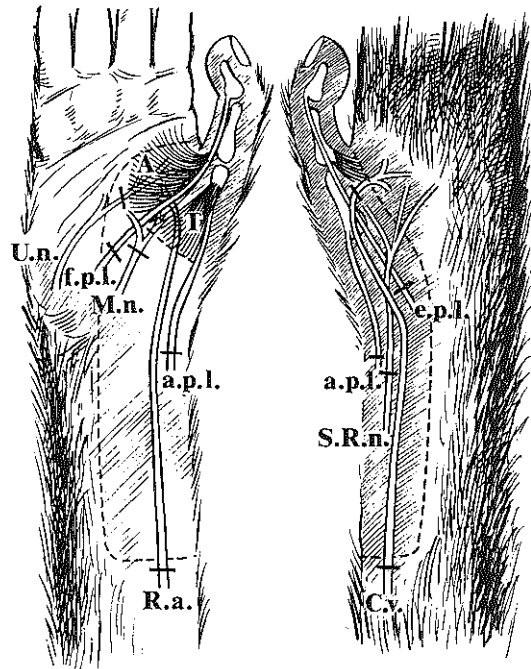
Subsequently, a custom-made thermoplastic splint was developed for the protection of the transplant following operation (ChII.C). One piece thermoplastic splints proved to be light, strong, easy to make, and inexpensive. Furthermore, they provided adequate immobilization and protection of the hand and allowed easy removal and reapplication for inspection. Complications from 39 splints, applied for 12 to 79 days, were minimal and transient (pressure ulcers: three; spontaneous removal of the splint: twice; twisting of the arm within the splint: once). They could all be managed by slight remolding of the splint.

To develop a highly immunosuppressive anti-rejection treatment another introductory study was designed (ChII.D). It was anticipated that the allogeneic hand transplant would suffer from severe rejection episodes as in two previous hand transplantation studies in the baboon where even high doses of CyA in combination with prednisolone could not prevent rejection of the CTA in the majority of the cases[20,21]. Nor could an increase in steroid treatment reverse all rejection episodes adequately. Monoclonal antibodies (MAbs), however, were known to be capable of selective reactivity with target structures that could vary from cell-surface markers to distinct molecules (like cytokines) (ChI.B.3.1.5). In preclinical experiments in the rodent and nonhuman primate as well as in the clinical setting, MAbs had proven to be capable of reversing rejection episodes (ChI.B.3.1.5). Most interestingly, MAbs specific for the pan T-cell marker CD3[a] had been demonstrated to be significantly better (kidney allograft rejection reversal rate of 94%) than conventional steroid treatment (reversal rate 75%)[22]. Furthermore, anti-CD3 MAbs have also been reported to be effective for the reversal of kidney and liver allograft rejection episodes that were nonreversible by high-dose steroids and/or anti-lymphocyte globuline[23,24]. It was hoped therefore, that by means of a combination of MAbs with immunosuppressive potency an effective therapy could be found for the treatment of CTA-rejection episodes.

In a skin transplantation study in the rhesus monkey, a combination of seven MAbs were tested respectively specific for the following cell surface markers: CD3 (pan T-cell), CD4 (helper/inducer T-cell subset), CD8 (cytotoxic/suppressor T-cell subset) and MHC class II (antigen presenting cells and activated T-cells)[b] (Fig.2). A toxicity-effect study in three monkeys which received doses ranging from 0.9 to 9 mg/kg revealed that 4.5 mg/kg induced maximal effect on lymphocyte subsets while side-effects were minimal and transient (gall expectoration once). Promisingly, in this dose, the combination of these MAbs seemed to

[a]CD = Cluster of Differentiation, as defined during the Leucocyte Typing Conferences in Paris, 1982; Boston, 1984; Oxford, 1986; and Vienna, 1989.

[b]MHC antigens = Major Histocompatibility Complex antigens (discussed in ChI.B.3.1.1)

have an additive effect on top of each individual effect of prolongation of skin allograft survival times. Mean allograft survival times could be prolonged from 8.3 (SD=0.7) to 19.3 (SD=1.3) days in a fully mismatched donor-recipient combination. Immunomodulatory effects of each individual MAb seemed to remain present when combined in a cocktail. In this respect the following phenomena were observed: coating (formation of a mantle of the cell by the injected MAbs), cell elimination (removal of the target cell) and antigen modulation (the target at which the injected MAb was aimed is removed from the cell surface: the cell is now naked as far as this surface structure is concerned, ChI,B.3.1.5). Noteworthy, approximately seven days after operation the appearance of anti-mouse-antibodies (AMA) neutralized the injected MAbs. This AMA-response is known to limit the beneficial use of MAbs. Alternatives to circumvent this major drawback of MAb-treatment were discussed in ChI,B.3.1.5.

In order to obtain maximal information on the immunological events that take place during the immune response against the allogeneic (radial side of the) hand, in a last introductory study 2 anti-rhesus and 28 anti-human MAbs were tested for immunohistochemical staining reactivity on frozen sections of rhesus monkey tissue (ChII,E). Out of these MAbs, 23 proved to be suitable for immunohistological studies of the following antigens: MHC class I, II-DR, -DQ, and -DP antigens; the leucocyte markers CD1, CD2, CD3, CD4, CD4, CD8, CD14, CD25 and CD57; and a proliferation associated nuclear antigen. Eventually, a selection of the five most relevant MAbs was used in our later studies for the comparison of intravascular and intra-allograft events (ChIII,B,ChIV,A) following transplantation.

With the knowledge gained from these introductory studies, four autologous *re*plantations of the partial hand were performed[25] in the rhesus monkey (ChII,C). Technical feasibility of the model could be demonstrated. By means of a 'sensiometer', electromyography, histology and function tests good motor and sensory recovery could also be demonstrated. The replanted thumb was again fully integrated in hand function. The contribution of the thenar muscle to the overall function of the thumb could, however, not be quantified very well. As a consequence of these results, permission was given by the ethical committee of the Primate Center TNO to continue the research project and perform the allogeneic *trans*plantations.

Technical feasibility of allogeneic transplantation of the radial side of the hand could be demonstrated (ChIII,A). In a consecutive series 14 transplantations were performed between a fully mismatched donor-recipient combination. 12 Transplantations were performed successfully. Two technical failures occurred due to irreversible vascular occlusion. This result supports data from the first and the only two previously published transplantation studies in the nonhuman primate, in which the total hand had been transplanted successfully

in the baboon[20,21].

Allograft recipients were divided into four treatment subgroups of three monkeys with successfully transplanted allografts per group, following a statistical design according to Fisher[26] (Table 1). This experimental design would enable us to economize on monkeys and evaluate the effect of preoperative third party blood transfusions as well as the value of the earlier mentioned MAb anti-rejection therapy. Preoperative blood transfusions were added to the research protocol for their known favorable effect on graft survival (ChI,B.3.1.3).

Various recent studies in nonhuman primates stressed the inevitability of using continuous high doses of CyA (20-48 mg/kg/day) to obtain prolonged survival of CTAs[20,21,27]. Therefore, in the present study, 25 mg/kg/day CyA in combination with a maintenance dose of steroids was used, yielding allograft survival times ranging between 21 and 179 days (Table 1). More specifically, CTA survival times were short in six monkeys (21-33 days) and long in the other six (79-179 days). Based on survival times of fully mismatched skin grafts in the rhesus monkey, it can be assumed that without this base line immunosuppression allografts would have been rejected within 7 to 10 days[28] (ChII,D).

By means of a specific radioimmuno-assay (RIA) it was demonstrated that whole blood CyA trough levels were above the minimal required concentration of 400 ng/ml[28] in 83% of all postoperative samples and in 92% of all samples after day five. CyA levels from day 5 to start of anti-rejection therapy did not differ significantly between long term and short term survivors. Median CyA levels were 861 (SD=272) and 676 (SD=256) ng/ml, respectively. Still, ten out of twelve recipients showed rejection of their allograft.

In general, monitoring of microvascular surgery, as for instance laser doppler flowmetry (LDF) and temperature recordings, can be a helpful tool in detecting vascular compromise, particularly if monitoring is performed continuously. As far as technical failures are concerned, one case could not be predicted as it occurred two days after surgery, the other had in retrospect low monitoring values directly after operation. With regard to the prediction of the onset of rejection after limb transplantation in the rat also the LDF-recordings were performed. In this study, falling LDF-values correlated well with the progress of rejection. However, a predictive value could not be established (ChII,A). In the rhesus monkey LDF-values appeared to be too variable and could not be assessed frequently enough, resulting in the inability to draw any conclusions with regard to the predictive parameter for the onset of rejection (ChIII,A).

Third party blood transfusions did not induce a significant difference in the time of onset of allograft rejection (Table 1). Blood transfusions in this study neither facilitated reversal of graft rejection nor had a significant effect on allograft survival (ChIII,A&B).

Table 1. Allogeneic transplantation of the radial side of the hand in the rhesus monkey; Effects of cyclosporine A, prednisone, monoclonal antibodies and third party blood transfusions on graft survival and obtained sensory and motor function recovery.

Monkey	Rejection Protocol	reversal	Graft survival times (days)	First sign of functional recovery[a] Motor	Sensory	Percentage of sensory recovery
2799	MAbs	-	79[c]	<79[d]	34	75
4023	&	+				
	transf.	+[b]	121[c]	31	41	91
2988		-	22[c]			
3992	MAbs	-	144	<72[d]	34	80
3439		+				
		+[b,e]	97[c]	46	56[f]	14
3308		no rejection	179[c]	42	64	100
2596	DAF	-	30			
2I	&	-	29[c]			
3310	transf.	no rejection	85[c]	56	29	84
1FU	DAF	-	33			
1550		-	33			
3212		-	21			

[a]functional recovery concerns long term survivors only (n=6 monkeys); [b]second rejection episode; [c]death, spontaneously or due to euthanasia; [d]first measurement with electromyography; [e]reversal of rejection was partial: skin on the palmar side of the thumb and thenar was rejected; [f]rejection episode at day 41. **Abbreviations: MAbs:** anti-rejection therapy consisted of a ten day course of a combination of seven monoclonal antibodies; **transf.:** three third party blood transfusions were given to the recipient, preoperatively; **DAF:** anti-rejection therapy consisted of a raise in steroid treatment; +: reversal; -: no reversal.

The absence of a protective effect of third party blood transfusions on CTA survival, could be due to the usage of high doses of CyA and DAF masking the expected effect. Alternatively, the difference in antigenicity of a CTA including skin compared to a kidney or heart allograft might have impaired an anticipated transfusion effect[29]. Possibly for the same reasons, retrospective analysis did not accord with the recent clinical evidence that heart and kidney allograft survival will be impaired by fully mismatched blood transfusions due to the induction of sensitization[30]. In this latter study graft survival could only be improved by pretransplant blood transfusion from blood donors sharing at least one HLA-DR antigen with the transfusion recipients.

Five out of the ten monkeys that showed rejection of their transplant, were treated with an increase in steroids. In none of these cases, could reversal of rejection be obtained (Table 1). The other half were treated with the combination of MAbs, described above. In two out of five monkeys this treatment reversed their episode of rejection. As a result MAb-anti-rejection therapy prolonged CTA survival significantly longer than conventional anti-rejection therapy with steroids (P=0.015, log-rank test) (ChIII,A&B). Also a second rejection episode could be successfully treated, using the same combination of MAbs. Most likely this was achievable because in this study, no neutralizing monkey-anti-mouse antibodies were formed in any of the monkeys in which CyA and DAF therapy was continued during and after reversal of allograft rejection (ChIII,B.3.4). Without CyA and DAF, the same MAbs used in a skin transplantation study, did induce an antibody response detectable from the 7th day after the first injection onwards (ChII,D).

In compliance with the skin transplantation study, the injected MAbs generated coating of all peripheral blood lymphocytes, cell elimination of mainly CD3+,CD8+ lymphocytes and CD3- and CD8-antigen modulation in each of the allograft recipients in which rejection of the radial side of the hand could be reversed (ChIII,B). The first two mentioned effects could also be observed within the allograft itself, though of shorter duration[c].

In all long term survivors, sensory as well as motor function recovery occurred (ChIII,C). Longitudinal monitoring showed that initial sensory return occurred after 41,8 and 43,8 days respectively (Table 1). The transplanted skin area, supplied by the median nerve, was reinnervated for an average of 74% (14-100%) as assessed by the sensiometer. Rejection had a negative effect on the sensibility as well as motor function. This could be demonstrated by a reduction of the skin area that would respond to sensory testing (provoking a withdrawal reflex after application of a small electrical current to the skin) and decrease of the amplitude

---

[c] For immunohistochemical staining reactivity with a pan-T cell marker a MAb specific for CD2 (and not CD3) was used.

of the compound motor action potential (CMAP) during electromyography. Reversal of rejection, however, established a clear increase of the skin area that was reactive to sensory nerve testing. Also increase to pre-rejection values of the amplitude of the CMAP was observed. Immunohistochemical staining for viable motor-end plates provided additional evidence for thenar muscle re-innervation. Further evidence was retrieved from a terminal experiment in one allograft recipient where direct stimulation of the perineurium of the median nerve in the forearm produced a clear CMAP at the heterologous thenar muscles. These data support the findings of the previous hand transplantation studies in the baboon, that host axons can grow into rejection-free but also into severely rejected (but still viable) histoincompatible tissue environment and re-innervate donor sensory receptors[31].

Despite these promising results, complications were noted (ChIII,D). Long term survivors lost weight ranging from 8 to 40% (average 20%) in relation to preoperative weight. Though kidney- and liver parameters were elevated in three and four monkeys respectively, no severe nephro- and/or hepatotoxicity was observed. Using similar high doses of CyA and steroids in man, more severe kidney and liver damage may occur[32]. Seven allograft recipients died during the experiment of which one was due to shock after the first injection of MAbs, three were due to posttransplantation lymphoproliferative (PTLP)-disorders and three were due to opportunistic bacterial infections (Table 1). Furthermore, one monkey that died from sepsis also had PTLP-disorder development at autopsy. Remarkably, similar doses of CyA and DAF were administered for periods of more than one year in baboons and no such side effects were reported[20,21]. A previous history of kidney donorship or testis radiation did not significantly correlate with occurrence of death or PTLP-disorder development. Addition of MAbs anti-rejection therapy to baseline immunosuppression did not correlate to incidence of death, significantly. However, it remains most likely that the occurrence of side effects is increased, directly related to the number and duration of immunosuppressive drugs administered[33].

Though our population is small, an enhanced predisposition is found to PTLP-disorder development (P=0.03, one-sided Fisher test) in the monkeys that received MAbs anti-rejection therapy. The MAbs used, were specifically aimed at lymphocytes, which might have precipitated lymphoid lesions, rather than other malignities. Alternatively, virus infections, notably Epstein-Barr[34] and Human T-Leukemia Virus (HTLV) infections[35], have a possible etiological link with the development of lymphoma. Apparently, only two CTA recipients, that showed PTLP-disorder development, were seropositive for antibody titers against Simian TLV (STLV) (ChIII,D). However, all four PTLP-disorders showed presence of STLV provirus at the DNA level in malignant tissue (ChIII,E). This implicates a possible etiological role of STLV in PTLP-disorder development in combination with additional

immunosuppressive therapy. However, the diversity of morphologic and phenotypic PTLP-disorder characteristics does not indicate a single etiology in the development of these disorders.

Clearly, the limitations of the currently used immunosuppressive treatments indicate that further research in this field is required before transplantation of the allogeneic hand in man should ever be performed. In this respect the immunosuppressive effect of two new MAbs specific for potent immunomodulatory cytokines was investigated in a rhesus monkey skin transplantation model (ChIV,A). Prophylactic treatment with an anti-interferon-γ or anti-tumor necrosis factor-α specific MAb alone did not prolong skin graft survival times. Neutralization of either one of these cytokines, was apparently not sufficient to suppress the immune response. However, treatment with the combination of these MAbs resulted in a modest but significant prolongation of allograft survival times. Compared to MAbs specific for MHC class II positive and CD8+ cells, which had mean skin graft survival time of 12.4 and 12.9 days[36] (ChII,D) respectively, the immunosuppressive effect of the combination of anti-interferon-γ and anti-tumor necrosis factor-α was equally strong.

Also, a skin transplantation study in the rat was performed to test the newly available drug FK-506 (ChI,B.3.1.6 & ChIV,B). Administration for a duration of 15 days of 0.1 mg FK-506 had a similar effect on skin graft survival as 5 mg CyA. Furthermore, in agreement with other experimental data it was found that minimally therapeutic doses of FK-506 act synergistically with suboptimal doses of CyA[37,38]. Recently, also in a clinical trial, oral administration of FK-506 in low doses was demonstrated to be an effective and safe immunosuppressant for liver, kidney and pancreas transplantation[39]. With regard to these data, it seems worthwhile to investigate the immunosuppressive potency of FK-506 in an allogeneic composite tissue model. Preliminary results on this topic also seem promising[40].

CONCLUSIONS

Technical aspects

Studies with respect to the anatomy of the hand of the rhesus monkey can support the assumption that data yielded from hand transplantation experiments in the rhesus monkey can be extrapolated to the human situation.

Technical feasibility of the research model could be demonstrated in the replants. Also allogeneic transplantation of the radial side of the hand could be realized technically in a rhesus monkey model. Wound healing was uneventful in both replants and transplants.

Monitoring devices like temperature measuring and laser doppler flowmetry could not provide a predictive value for vascular occlusion nor for onset of rejection in this model.

Functional aspects

The thumb was again fully integrated in hand function following *autologous replantation* of the radial side of the hand. Very good function recovery could be established in these monkeys. Also following *allogeneic trans*plantation of the radial side of the hand sensory and motor function recovery occurred. Rejection caused a decrease in functional recovery, however this increased again if rejection was reversed.

Evaluation of sensory and motor function recovery could only be performed during a half year postoperatively. Nevertheless, sensory recovery reached near normal levels in the non-rejected allografts when compared to the contralateral side.

With respect to motor recovery the amplitude of the compound motor action potentials of the thenar muscles as well as the distal thenar muscle latency time in non-rejected allografts approached the values of the same parameters on the contralateral side.

Immunohistochemical staining demonstrated vital motor-end plates as evidence for motor reinnervation.

Immunological aspects

In skin transplantation studies in the rhesus monkey the immunosuppressive potency was tested by a combination of seven monoclonal antibodies (MAbs) specific for the following cell surface structures: CD3 (=pan T-cell), CD4 (helper/inducer T cell-subset) and CD8 (cytotoxic/suppressor T-cell subset). In combination these MAbs appeared to possess an additive effect on top of their individual effect to prolong allograft survival times.

Continuous high doses of cyclosporine A and prednisone enabled long term graft survival in six out of twelve allograft recipients. The aforementioned MAbs therapy could reverse rejection prolonging allograft survival times significantly longer than steroid anti-rejection treatment. MAbs therapy induced a vigorous elimination of mainly the CD2+, CD3+ (=pan T-cell), and CD8+ (=cytotoxic/suppressor T-cell subset) lymphocyte subsets in the peripheral blood as well as in the allograft.

Despite these and afore mentioned results the currently used immunosuppressive regimen also had some major complications: seven rhesus monkeys died during experiment. In four of these monkeys a posttransplantation lymphoproliferative (PTLP)-disorder developed and in all of these cases the Simian T-Leukemia Virus (STLV)-provirus was detected in the DNA of the lymphoproliferative tissue.

The presence of STLV-provirus in all four PTLP-disorders implicates a possible etiologic role of STLV in tumor development, especially when combined with high doses of immunosuppressive therapy. It should also be noted that the addition of MAbs to base-line

immunosuppressive therapy correlated (weakly) to PTLP-disorder development.

In the context of the search for necessary improvements to control rejection and minimize side effects, a synergistic effect was found firstly, between MAbs specific for the cytokines interferon-gamma and tumor necrosis factor-alpha and secondly, between cyclosporine A and the new immunosuppressive drug FK-506.

Prospects

Transplantation of the allogeneic hand seems to be closer to reality, since some promising results were obtained in technical, functional and immunological aspects. However, as a consequence of the fact that using high doses of the best immunosuppressive agents available today, still seven monkeys died prematurely, it is therefore necessary to seek a more effective and less toxic regimen before transplantation of the (total) hand can be performed in man.

Once such an agent or combination of agents becomes available than most likely the indication for functional and esthetic reconstruction by the use of allogeneic tissue will be present.

Referenties

1. Merion RM, White DJG, Thiru S, et al. Cyclosporine: Five years' experience in cadaveric renal transplantation. N Eng J Med 1984; 310: 148.
2. Hors J, Busson M, Bouteiller AM, Raffoux C, Betuel H, Fauchet R, Tongio MM, Mercier P, Lepetit JC, Dausset J: Dissection of the respective importance of HLA-A,B,DR matching in 3,789 prospective kidney transplants. Transpl Proc 1987; 19: 687.
3. McGregor CGA, Oyer PE, Shumway NE. Heart and heart-lung transplantation. Prog Allergy 1986; 38: 346.
4. Starzl TE, Iwatsuki S, Shaw BW Jr, et al. Orthotopic liver transplantation in 1984. Transplant Proc 1985; 17: 250.
5. Ringden O. Cyclosporine in allogeneic bone marrow transplantation. Transplantation 1986; 42: 445.
6. Brooks JR: Where are we with pancreas transplantation? Surgery 1989; 106: 935.
7. Watson AJ, Lear PA: Current status of intestine transplantation. Gut 1989; 30: 1771.
8. Ding YL, Han CM: Recent advances in burn wound management in China. Actua Chir Plast 1989; 31: 84.
9. Schwind JV: Homotransplantation of extremities of rats. Radiology 1962; 78: 806-809.
10. Doi K: Homotransplanation of limbs in rats: a preliminary report on an experimental study with nonspecific immunosuppressive drugs. Plast Reconstr Surg 1979; 64: 613-624.
11. Black KS, Hewitt CW, Fraser LA, et al: Cosmas and Damian in the laboratory. N Eng J Med 1982; 306: 368-369.

12. Furnas DW, Black KS, Hewitt CW, et al: Indefinite composite tissue (limb) allograft survival in rats with cyclosporine : Possible furure implications in plastic and reconstructive surgery. Transpl Proc 1983; 15: 3063.

13. Hewitt CW, Black KS, Fraser LA, et al: Cyclosporine A (CyA) is superior to prior donor-specific blood (DSB) transfusion for the extensive prolongation of rat limb allograft survival. Transpl Proc 1983; 15: 514-517.

14. Fritz WD, Swartz WM, Rose S, Futrell JW, Klein E: Limb allografts in rats immunosuppressed with cyclosporine A. Ann Surg 1984; 199: 211.

15. Black KS, Hewitt CW, Fraser LA, et al: Composite tissue (limb) allografts in rats. II. Indefinite survival using low-dose cyclosporine. Transplantation 1985; 39: 365.

16. Hewitt CW, Black KS, Dowdy SF, et al: Composite tissue (limb) allografts in rats. III. Development of donor-host lymphoid chimeras in long-term survivors. Transplantation 1986; 41: 39.

17. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21: 3191.

18. Jonker M, Goldstein G, Balner H: Effects of in vivo administration of monoclonal antibodies specific for human T cell subpopulatioons on the immune system in a rhesus monkey model. Transplantation 1983; 35: 521.

19. Jonker M: The importance of nonhuman primates for preclinical testing of immunosuppressive monoclonal antibodies. Seminars in Immunology, 1990, (in press).

20. Daniel RK, Egerszegi EP, Samulack DD, Skanes SE, Dykes RW, Rennie WRJ: Tissue transplants in primates for upper extremity reconstruction: a preliminary report. J Hand Surg 1986; 11A:1.

21. Stark GB, Swartz WM, Narayanan K, Moller AR: Hand transplantation in baboons. Transplant Proc 1987; 19: 3968.

22. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. New Eng J Med 1985; 313 (6): 337.

23. Ponticelli C, Rivolta E, Tarantino A, Egidi F, Banfi G, DeVecchi A, Montagnino G, Vegeto A: Clinical experience with ORTHOCLONE OKT3 in renal transplantation. Transpl Proc 1986: 18: 942.

24. Starzl TE, Fung JJ. ORTHOCLONE OKT3 in treatment of allografts rejected under Cyclosporine-steroid therapy. Transpl Proc 1986; 18: 937.

25. Hovius SER, Stevens HPJD, Jonker M, van der Meulen JC: Partial hand transplantation in the rhesus monkey: Phase I: Technical feasibility of the model. Annual Report REP-TNO, 1987: 197.

26. Armitage P, Berry G. Factorial design. In: Armitage P eds. Statistical methods in medical research. London, UK: Blackwell Scientific Publications, 1987: 227.

27. Skanes SE, Samulack DD and Daniel RK. Tissue transplantation for reconstructive surgery. Transpl Proc 1986; 18(4): 898.

28. Vreeswijk W, Balner H. Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 1980; 30 (3): 196.

29. Hewitt CW, Black KS, Stenger JA, Go C, Achauer BM, Martin DC. Comparison of kidney, composite tissue and skin allograft survival in rats prolonged by donor blood and concomitant limited cyclosporine. Transpl Proc 1988; 20 (3,S3): 1110.

30. Lagaaij EL, Henneman IPH, Ruigrok M, etal: Effect of one-HLA-DR-antigen-matched and completely HLA-DR-mismatched blood transfusions on survival of heart and kidney allografts. N

Engl J Med 1989; 321: 701.

31. Samulack DD, Munger BL, Dykes RW, Daniel RK: Neuroanatomical avidence of reinnervation in primate allogarfted (transplanted) skin during cyclosporine immunosuppression. Neurosci Lett 1986; 72: 1.

32. Scott JP. Higenbottam TW. Med Toxicol 1988; 3: 107.

33. Penn I: Cancers following cyclosporine therapy. Transplantation 1987; 43: 32.

34. Hanto DW, Gajl-Peczalska KJ, Frizzera G, etal: Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occuring after renal transplantation. Clinical, pathological, and virological findings and implications for therapy. Ann Surg 1983; 198: 356.

35. Clark JW, Robert-Guroff M, Ikehera O, Henzen E, Blattner WA: Human T-cell leukemia-lymphoma virus type 1 and adult T-cell leukemia-lymphoma in Okinawa. Cancer Res 1985; 45: 2849.

36. Jonker M, Nooij FJM, Suylichem P van, Neuhaus P, Goldstein G. The influence of OKT8F treatment on allograft survival in rhesus monkeys. Transplantation 1986; 41(4): 431.

37. Murase N, Todo S, Lee PH et al. Transplant Proc 19:71,1987

38. Zeevi A, Duquesnoy R, Eiras G et al.: Transplant Proc 20:220, 1988

39. Starzl TE, Todo S, Fung J, etal: FK-506 for liver, kidney and pancreas transplantation. Lancet 1989; 2: 1000.

40. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21(1): 3191-3193.

HOODFSTUK VI: DISCUSSIE EN SAMENVATTING

S.E.R. Hovius & H.P.J.D. Stevens

Dit 'dubbel-proefschrift' beschrijft onderzoek over de technische, functionele en immunologische aspecten van allogene[a] transplantatie van de (radiaire[b] zijde van de) hand, in de rhesusaap.

Onderzoek op dit gebied werd geïnitieerd door de vraag van patiënten en/of ouders of het probleem van een niet functionerende of afwezige hand eventueel kon worden opgelost door allogene transplantatie. Wat de onderliggende oorzaak ook moge zijn, traumatisch, aangeboren of oncologisch, zelfs de meest geavanceerde prothese kan het verlies van sensibiliteit en motoriek niet vervangen. Tegenwoordig kan klinisch bijna ieder orgaan worden getransplanteerd uiteenlopend van nier[1,2], hart (soms in combinatie met de longen)[3], lever[4], beenmerg[5], alvleesklier[6], ingewanden[7], zelfs huid[8]. Met name dankzij de ontdekking van Cyclosporine A (CyA) (Chapter I, section B.3.1.4: ChI.B.3.1.4) kunnen redelijke tot zeer goede transplantaat- en patiëntoverlevingstijden worden verkregen[1-7]. Bovendien zijn sinds de zestiger jaren microchirurgische technieken ter beschikking gekomen die het mogelijk hebben gemaakt zeer kleine vaten en zenuwen te hechten (ChI.A.1). Op grond van deze ontwikkelingen is experimenteel onderzocht of handtransplantatie met langdurig overleven en behoud van functie mogelijk is.

Talrijke studies zijn verricht die zich concentreerden op de haalbaarheid een combinatie van meerdere verschillende weefsels, zoals bot, pezen, zenuwen, vaten en huid (hierna te noemen een CTA: 'composite tissue allograft') te transplanteren (ChI). Het betrof dan voornamelijk achterpoottransplantaties bij de rat[9-17]. Uit laatstgenoemde experimenten kunnen technische, functionele en immunologische aspecten die van belang zijn naar voren komen. In dit opzicht kon door onze groep worden aangetoond dat fluctuaties van perifere bloedgassen, bloedsuiker- en melkzuurspiegels het moment van afstoting van een CTA niet voorspellen (ChII.A). Wel bleek dat de flux (aantal per tijdseenheid) van perifere bloedcellen door de huid van een CTA, gemeten met een laserdopplerflow-(LDF)-meter[c], daalde bij een toename van de transplantaatafstoting. Echter, ook deze LDF-metingen hadden geen voorspellende waarde ten aanzien van het begin van afstoting, waarschijnlijk door de onmogelijkheid om continu te meten. De eerste zichtbare tekenen van afstoting waren steeds

---

[a]afkomstig van een ander individu van dezelfde species

[b]aan de kant van de radius, het spaakbeen cq. de kant van de duim

[c]De LDF-meter is een apparaat dat de huiddoorbloeding meet met behulp van een laserstraal op basis van het doppler-effect.

oedeemvorming[d], haaruitval en epidermolysis[e].

Voor extrapolatie naar de situatie bij de mens hebben studies in knaagdieren duidelijk hun beperkingen, zowel vanuit technisch en functioneel alsook vanuit immunologisch oogpunt. Wanneer ooit tot transplantatie van de (totale) hand bij de mens wordt overgegaan dan zal informatie over de technische haalbaarheid, de mate van afstoting en het herstel van functie van eminent belang zijn.

Bij voorkeur dient een nonhumane primaat te worden gebruikt voor het preklinisch testen van deze aspecten van allogene handtransplantatie vanwege de overduidelijke anatomische, functionele, immunologische en fylogenetische[f] overeenkomsten tussen de aap en de mens. In verband hiermee is de aap een beter proefdier dan andere dieren om de werking en bijwerking van immunosuppressieve middelen en monoclonale antilichamen (MAbs) te testen[18,19].

Figuur 1. Het transplantaat. De gestippelde lijn markeert de getransplanteerde radiaire zijde van de hand. Handpalm: A = adductor spier; T = thenar spier; U.n. = ulnaire zenuw; M.n. = mediane zenuw; r.a. = radiaire arterie; Pezen: f.p.l. = flexor pollicis longus; a.p.l. = abductor pollicis longus. Handrug: s.r.n. = superficiale radiaire zenuw; c.v. = cephalica vene; Pezen: e.p.l. = extensor pollicis longus; a.p.l. = abductor pollicis longus.

Op grond van bovengenoemde aspecten van allogene handtransplantatie was het ons doel dit onderzoek te verrichten in een rhesusaapproefdiermodel. [def]

---

[d]onderhuidse vloeistofophoping

[e]loslating van de opperhuid

[f]ontwikkeling van de soort gedurende de evolutie

In het experimentele model werd gebruik gemaakt van een transplantaat dat bestond uit de radiaire zijde van de hand tesamen met de radiaire onderarmslap (Fig.1, ChI.C.1.2). Uit ethisch oogpunt werd een kleine, onafhankelijk functionerende eenheid van de hand gekozen. Eén van de inleidende studies bewees inderdaad dat de anatomie van het transplantaat van de rhesusaap zeer te vergelijken was met de situatie bij de mens (ChII.B). De rhesusaap leeft gecombineerd op de grond en in de bomen. Door dit leefpatroon heeft de rhesusaap een beter functionerende duim nodig dan bijvoorbeeld een slingeraap. De rhesusaap is in staat tot oppositie, echter niet zo goed als de mens. De duim van de rhesusaap was in verhouding korter dan de duim bij de mens. Belangrijke verschillen waren dat de lange buiger (musculus flexor pollicis longus) naar de duim zich pas afsplitste van de andere buigers op het niveau van de pols. Ten gevolge hiervan kan de duim minder onafhankelijk buigen. Tevens bleek dat bij de rhesusaap de korte strekker (musculus extensor pollicis brevis) ontbreekt, waardoor stabilisatie van het metacarpophalangeale gewricht[g] van de duim minder optreedt bij grijpbewegingen. Een belangrijke overeenkomst was dat de duimmuisspieren van de rhesusaap in aanleg gelijk waren aan die van de mens. De zenuwen naar de duim hadden een vrijwel identiek verloop.

Vervolgens werd een op maat gemaakte spalk ontwikkeld voor de bescherming van het transplantaat na operatie (ChII.C). Spalken van thermoplast uit één stuk bleken licht, sterk, gemakkelijk te maken en goedkoop te zijn. Bovendien verschaften zij een adequate immobilisatie en bescherming van de hand. Ze konden tevens gemakkelijk verwijderd en teruggeplaatst worden bij wondinspectie. De spalken waren geplaatst voor periodes van 12 tot 79 dagen. De complicaties bij de 39 spalken bleken minimaal (ulcera: driemaal; spontaan uittrekken van de spalk: tweemaal; draaien van de arm in de spalk: éénmaal). Ze konden alle adequaat worden behandeld door het aanbrengen van lichte veranderingen aan de spalk.

Om een sterk immunosuppressieve anti-afstotingsbehandeling te ontwikkelen was een andere inleidende studie ontworpen (ChII.D). Er werd namelijk verwacht dat de allogene handtransplantaten episoden van zware afstoting te verduren zouden krijgen. Immers zoals bleek uit twee vorige studies van handtransplantatie bij de baviaan konden zelfs zware doses CyA in combinatie met steroïden afstoting van het CTA in de meerderheid van de gevallen niet voorkomen[20,21]. Tevens kon een verhoging van de steroïdenbehandeling niet alle afstotingsepisoden adequaat omkeren. MAbs echter, kunnen selectief reageren met doelwitten die uiteen kunnen lopen van celoppervlaktestructuren tot bepaalde moleculen

---

[g]het gewricht tussen de middenhandsbeenderen en de vingerkootjes

(zoals cytokinen[h]) (ChI.B.3.1.5). In preklinische experimenten bij het knaagdier en de nonhumane primaat alsook bij klinische experimenten hebben MAbs bewezen afstotings-episoden te kunnen omkeren (ChI.B.3.1.5). Interessant genoeg bleken MAbs, specifiek voor de 'algemene T-cel marker' CD3[i], hiertoe significant beter in staat te zijn (niertransplantaat-afstotings-omkerings-percentage 94%) dan conventionele behandeling met steroïden (afstotings-omkerings-percentage 75%)[22]. Bovendien is gerapporteerd dat anti-CD3 MAbs effectief zijn bij de omkering van nier- en leverafstotingsepisoden die niet meer reageerden op hoge doses steroïden en/of anti-lymfocyten globuline[23,24]. Om deze reden werd gehoopt dat door middel van een combinatie van meerdere MAbs met immunosuppressieve potentie een adequate therapie gevonden kon worden voor de behandeling van CTA-afstotingsepisoden.

In een huidtransplantatiestudie bij de rhesusaap werd een combinatie van zeven MAbs getest die respectievelijk specifiek waren voor de volgende celoppervlaktestructuren: CD3 (pan T-cel), CD4 (helper/inducer T-cel subset), CD8 (cytotoxische/suppressor T-cel subset) en MHC klasse II (antigeen presenterende cellen en geactiveerde T-cellen)[j] (Fig.2). Een toxiciteits-effect studie met deze combinatie van MAbs bij drie rhesusapen die doses ontvingen variërend van 0.9 tot 9 mg/kg onthulde dat 4.5 mg/kg maximale effecten teweeg bracht op de lymfocyten-subsets terwijl de neveneffecten minimaal en van voorbijgaande aard bleken te zijn (eenmalig werd gal gebraakt). Veelbelovend bleek dat in deze dosering de combinatie van deze MAbs een additief effect bleek te hebben om de transplantaat-overleving te verlengen. Gemiddelde huidoverlevingstijden konden worden verlengd van 8.3 (SD=0.7) tot 19.3 (SD=1.3) dagen in een volledig gemismatchte donor-gastheer combinatie.

Immunomodulatoire effecten van ieder individueel MAb leken aanwezig te blijven bij het combineren tot een cocktail. In dit opzicht werden gezien: 'coating' (bedekken van het celoppervlak door de ingespoten MAbs), 'cel-eliminatie' (verwijdering van de doelwit-

---

[h]immuno-actieve stoffen die door bepaalde cellen als T-lymfocyten en macrofagen als reactie op bepaalde prikkels (o.a. antigenen) worden geproduceerd.

[i]CD = Differentiatie-cluster zoals deze zijn getypeerd gedurende de 'Leucocyt Typerings Conferenties' in Parijs, 1982; Boston, 1984; Oxford, 1986; en Wenen, 1989. Deze 'oppervlakte-antennes' komen voor op allerlei witte bloedcellen (leucocyten); het gedeelte kleine witte bloedcellen met ronde of ovale kern en smalle zoom protoplasma (lymfocyten) is onder te verdelen in: lymfocyten die antilichamen produceren (B-cellen) en lymfocyten die de cellulaire immuniteit verzorgen (T-cellen).

[j]MHC = 'Major Histocompatibility Complex', bediscussieerd in ChI.B.3.1.1.

cellen) en 'antigeen-modulatie' (het doelwit waartegen de ingespoten MAbs gericht waren is van het celoppervlak verwijderd: de cel is 'naakt' voor wat betreft deze oppervlakte-structuur) (ChI.B.3.1.5). Opgemerkt dient te worden dat ongeveer zeven tot negen dagen na eerste toediening de verschijning van anti-muis-antilichamen (AMA) de ingespoten MAbs (die van muizen-origine waren) neutraliseerden. Van de AMA-respons is bekend dat deze het werkzame effect van MAbs teniet doet. Alternatieven om deze beperking van het gebruik van MAbs te voorkomen zijn besproken in ChI.B.3.1.5.

Om maximale informatie te verkrijgen over de immunologische gebeurtenissen die plaats vinden gedurende de immuunrespons tegen de allogene (radiaire zijde van de) hand, werden in een laatste inleidende studie twee anti-rhesus en 28 anti-mens MAbs getest op hun immunohistochemische kleuringsactiviteit op diepgevroren coupes van rhesusaapweefsel (ChII.E). Van deze MAbs bleken 23 stuks geschikt te zijn voor de immunohistochemische studie van de volgende antigenen: MHC klasse I, II-DR, -DQ, en -DP antigenen; de leucocyt-markers CD1, CD2, CD3, CD4, CD8, CD14, CD25 en CD57; en een kern-anti-geen geassocieerd met celdeling. Uiteindelijk is in onze latere studies een selectie van de vijf meest relevante MAbs gebruikt voor de vergelijking van post-transplantatie-gebeurtenissen binnen de bloedbaan en die buiten de bloedbaan in het transplantaat zelf (ChIII.B & ChIV.A).

Met de kennis verkregen uit deze vijf inleidende studies, werden bij de rhesusaap vier autologe *re*plantaties van de radiaire zijde van de hand verricht (ChII.C). De technische haalbaarheid van het proefdiermodel kon worden aangetoond. Door middel van een "sensiometer"[k], electromyografie, histologie en functietesten kon eveneens een goed motorisch en sensibel herstel worden aangetoond. De duim was weer volledig geïntegreerd in de handfunctie na replantatie, het aandeel van de duimmuisspieren in de totale samengestelde beweging van de duim kon echter niet goed gequantificeerd worden. Naar aanleiding van deze gegevens werd het door de ethische commissie van het Primaten Centrum TNO toegestaan te continueren met allogene *trans*plantaties.

Eveneens kon de technische haalbaarheid van transplantatie van de radiaire zijde van de hand in de allogene proefopzet worden aangetoond (ChIII.A). In een opeenvolgende serie werden 14 transplantaties verricht tussen volledig gemismatchte donoren en ontvangers; hiervan waren 12 succesvol. Twee technische mislukkingen waren te wijten aan vaat-afsluiting in de direct postoperatieve fase. Dit resultaat ondersteunt data van de eerste en enige twee voorafgaande transplantatiestudies bij de nonhumane primaat, waarbij de totale

---

[k]Apparaat waarmee een zeer lichte stroomstoot gegeven werd op de huid. Bij gevoelsherstel van het huidgebied volgde een terugtrek reactie van de arm.

hand succesvol kon worden getransplanteerd bij de baviaan[20,21].

De transplantaat-ontvangers werden verdeeld in vier behandelingsgroepen, ieder bestaande uit drie apen met een succesvol getransplanteerde radiaire zijde van de hand per groep (Tabel 1). Deze factoriële 'proefopzet van Fisher'[26] stelde ons in staat economisch verantwoord met het aantal benodigde apen om te gaan en maakte simultane evaluatie mogelijk van zowel het effect van preoperatieve derde partij bloedtransfusies[1] als de waarde van de eerder genoemde MAbs anti-afstotingstherapie. Preoperatieve bloedtransfusies werden toegevoegd aan het onderzoeksprotocol omdat bekend is dat zij een gunstig effect op de transplantaatoverleving kunnen hebben (ChI,B.3.1.3).

Meerdere recente studies bij de nonhumane primaat hebben de noodzaak benadrukt om continu hoge doseringen van CyA te gebruiken (20-48 mg/kg/dag) om verlengde overleving van CTA's te verkrijgen[20,21,27]. Zodoende werd in de huidige studie 25 mg/kg/dag CyA gebruikt in combinatie met een onderhoudsdosering steroïden. Dit leverde transplantaat-overlevingstijden op die uiteenliepen van 21 tot 179 dagen (Tabel 1). De CTA-overle-vingstijden waren kort bij zes apen (21-33 dagen) en lang bij de overige zes (79-179). Gebaseerd op de overlevingstijden van volledig gemismatchte huidtransplantaten bij de rhesusaap kan worden aangenomen dat zonder basis-immunosuppressie de allotransplantaten binnen 7 tot 10 dagen zouden zijn afgestoten[28] (ChII,D).

Door middel van een specifieke radioimmuno-assay (RIA) werd aangetoond dat de volbloed-dalspiegels van CyA boven de minimaal vereiste concentratie van 400 ng/ml waren[28] in 83% van alle postoperatieve monsters en in 92% van alle monsters na dag 5. CyA-dalspiegels tussen dag 5 en de dag van het begin van afstotingsbehandeling verschilden niet statistisch significant tussen lange en korte transplantaatoverlevers. Mediaan CyA-dalspiegels waren respectievelijk 861 (SD=272) en 676 (SD=256) ng/ml. Desalniettemin vertoonden tien uit twaalf rhesusapen afstoting van hun transplantaat.

In het algemeen is het monitoren na microvasculaire operaties door bijvoorbeeld laserdopplerflow-(LDF)-metingen en temperatuurregistratie, een zinnige methode om vasculaire problemen vroegtijdig te detecteren, met name als de monitoring continu wordt verricht. Wat betreft de twee technische mislukkingen kon in één geval vaatafsluiting niet worden voorspeld, aangezien deze pas twee dagen na operatie optrad. In het tweede geval bleek, achteraf bezien, dat de meetwaarden direct na operatie reeds laag waren.

---

[1] hiervan is sprake indien het bloed afkomstig is van bloeddonoren waarvan het MHC volledig verschilt van het MHC van zowel de transplantaatdonor als van dat van de transplantaat/bloedontvanger.

Tabel 1. Allogene transplantatie van de radiaire zijde van de hand bij de rhesusaap; Effecten van cyclosporine A, prednison, monoclonale antilichamen en preoperatieve derde-partij-bloedtransfusies op transplantaatoverleving en verkregen sensibel en motorisch herstel.

Aap	Rejectie Protocol	omkering van afstoting	transplantaat overlevingstijd (dagen)	Eerste teken van functioneel herstel[a] motorisch	sensibel	Percentage sensibel herstel
2799	MAbs	-	79[c]	<79[d]	34	75
4023	&	+				
	transf.	+[b]	121[c]	31	41	91
2988		-	22[c]			
3992	MAbs	-	144	<72[d]	34	80
3439		+				
		+[b,e]	97[c]	46	56[f]	14
3308		geen afstoting	179[c]	42	64	100
2596	DAF	-	30			
21	&	-	29[c]			
3310	transf.	geen afstoting	85[c]	56	29	84
1FU	DAF	-	33			
1550		-	33			
3212		-	21			

[a]functioneel herstel betreft alleen de lange overlevers (n=6 apen); [b]tweede afstotingsperiode; [c]dood, spontaan of ten gevolge van euthanasie; [d]eerste meting met electromyograaf; [e]afstoting werd slechts gedeeltelijk omgekeerd: de huid aan de palmaire zijde van duim en thenar werd afgestoten; [f]afstotingscrisies op dag 41. **Afkortingen: MAbs:** anti-rejectie therapie bestaat uit een 10-daagse kuur met de combinatie van 7 MAbs; **transf.:** drie derde-partij-bloedtransfusies werden preoperatief gegeven aan de ontvanger; **DAF:** anti-rejectie therapy bestond uit een verhoging van de prednison doses; +: omkering; -: geen omkering van afstoting.

Ten aanzien van het voorspellen van begin van afstoting werden bij de allogene extremiteitstransplantaties bij de rat o.a. LFD-metingen gebruikt. In deze studie correleerde de daling van de LDF-waarden goed met het optreden van afstoting, echter een predictieve waarde kon niet worden vastgesteld (ChII,A). In de rhesusaap bleken de LDF-metingen te variabel en kon niet vaak genoeg gemeten worden, zodat geen conclusies konden worden getrokken uit dit onderzoek betreffende de voorspellende waarde voor begin van afstoting (ChIII, A).

Derde-partij-bloedtransfusies induceerden geen significant verschil in het moment van begin van transplantaatafstoting (Tabel 1). Bloedtransfusies bleken noch omkering van afstoting te vergemakkelijken, noch hadden ze een significant effect op de transplantaat-overlevingstijden in deze studie (ChIII,A&B). De afwezigheid van een beschermend effect van derde-partij-bloedtransfusies op de CTA-overleving kan te wijten zijn aan het gebruik van hoge doses CyA en steroïden die het verwachte effect zouden hebben gemaskeerd. Anderzijds kan het verschil in antigeniteit[m] van een CTA met huid vergeleken met een nier- of harttransplantaat het bloedtransfusie-effect teniet hebben gedaan[29]. Mogelijk vanwege dezelfde redenen bleek retrospectieve analyse niet overeen te komen met het recent beschreven klinische bewijs dat nier- en harttransplantaatoverleving verslechterd zullen worden door volledig gemismatchte bloedtransfusies ten gevolge van sensitisatie[30]. In deze klinische studie bleek transplantaatoverleving alleen te worden verbeterd door pre-transplantatiebloedtransfusies die kwamen van donoren die ten minste een HLA-DR antigeen deelden met de bloedtransfusie-ontvangers.

De helft van de tien rhesusapen die een afstotingsreactie van hun transplantaat lieten zien, werd behandeld met een verhoging van de steroïdendoses. In geen van deze gevallen kon omkering van de afstoting worden bewerkstelligd (Tabel 1). De andere helft werd behandeld met de hierboven beschreven combinatie van MAbs. Bij twee van de vijf apen werd met deze behandeling de rejectie-episode omgekeerd (ChIII,B.3.4). Door de MAbs-anti-afstotings-therapie werd de CTA-overlevingstijd significant sterker verlengd dan het geval was met de conventionele anti-afstotingsbehandeling door steroïden (P=0.015, log-rank test) (ChIII,A&B). Een tweede episode van transplantaatafstoting kon eveneens succesvol worden behandeld, gebruikmakend van dezelfde combinatie van MAbs. Zeer waarschijnlijk was dit mogelijk vanwege het feit dat, in deze studie, geen neutraliserende aap-anti-muis-antilichamen waren gevormd in die apen waarbij de CyA en steroïdentherapie was gecontinueerd tijdens en na de omkering van de transplantaatafstoting. Zonder CyA en

---

[m]het vermogen als antigeen (stof die het organisme aanzet tot de produktie van antilichamen) te werken

steroïden induceerden dezelfde MAbs in een huidtransplantatiestudie wel degelijk een antilichaamrespons. Deze was detecteerbaar vanaf de zevende dag na de eerste injectie met MAbs (ChII,D).

De ingespoten MAbs bewerkstelligden coating van alle perifere bloedcellen, cel-eliminatie van hoofdzakelijk CD3+ en CD8+ lymfocyten en modulatie van CD3- en CD8-antigenen bij ieder van de CTA-ontvangers waarbij afstoting kon worden omgekeerd. Dit was ook het geval in de eerder verrichte huidtransplantatiestudie (ChIII,B). De eerste twee genoemde effecten konden met immunohistochemische kleuringen eveneens worden geobserveerd in de allotransplantaten zelf, zij het dat ze korter duurden[n].

Bij alle lange transplantaat overlevers trad zowel herstel van gevoel als spierfunctie op (ChIII,C). Longitudinaal onderzoek toonde aan dat tekenen van eerste herstel van gevoel en spierfunctie respectievelijk na 41,8 en 43,8 dagen optraden (Tabel 1). Het getransplanteerde huidgebied, verzorgd door de nervus medianus was gemiddeld voor 74% (14-100%) gereïnnerveerd, gemeten met behulp van de sensiometer. Afstoting had een negatief effect op zowel sensibiliteit als motoriek. Dit kon worden aangetoond door een verkleining van het gebied dat reageerde op sensibiliteitsonderzoek en een verlaging van de amplitude van het samengesteld ('compound') motor-actie potentiaal (CMAP) van de duimmuisspieren tijdens electromyografie. Omkering van afstoting echter, liet een duidelijk herstel zien van het huidgebied dat reageerde op sensibiliteitsonderzoek. De waarden van de amplitudes van de CMAP van de duimmuis spieren waren eveneens reversibel. Immunohistochemische kleuring van vitale motor-eindplaatjes leverde additioneel bewijs voor reïnnervatie van de duimmuisspieren. Extra ondersteuning hiervoor was afkomstig uit een terminaal experiment bij een allotransplantaat-ontvanger waarbij directe stimulatie op het perineurium van de nervus medianus[o] in de onderarm een duidelijk CMAP produceerde afkomstig van de allogene duimmuisspieren. Deze data geven aan dat gastheer-axonen sensibiliteitsreceptoren van de donor kunnen reïnnerveren[31].

Ondanks deze veelbelovende resultaten traden ook complicaties op (ChIII,D). Lange overlevers daalden 8 tot 40% (gemiddeld 20%) in gewicht ten opzichte van voor de operatie. Alhoewel nier- en leverwaarden verhoogd waren in respectievelijk drie en vier gevallen, trad

---

[n] Voor een immunohistochemische kleuringsreactie met de algemene T-cel marker werd een MAb gebruikt specifiek voor CD2 (en niet CD3).

[o] de in het midden van de pols gelegen zenuw die tevens de belangrijkste zenuw was bij het tot stand komen van sensibel en motorisch herstel van het transplantaat.

geen ernstige nier- en/of levertoxiciteit op. Indien vergelijkbare doses CyA en steroïden bij de mens worden gebruikt, dan kunnen weldegelijk ernstiger schade aan de nier en lever worden verwacht[32]. Zeven allotransplantaat-ontvangers overleden tijdens experiment (Tabel 1). Eén ten gevolge van shock na de eerste injectie van MAbs, drie ten gevolge van posttransplantatie lymfoproliferatieve (PTLP)-afwijkingen en drie ten gevolge van opportunistische bacteriële infecties. Eén van de apen die overleed aan een bacteriële infectie bleek bij autopsie ook een PTLP-afwijking te hebben ontwikkeld. Opmerkelijk is dat bij bavianen vergelijkbare doses van CyA en steroïden gedurende perioden langer dan een jaar zijn gegeven en dat geen van dit soort neveneffecten optrad[20,21]. De voorgeschiedenis, zoals nierdonorschap of testisbestraling bleek niet te correleren met het optreden van dood of PTLP-afwijkingen. Toevoeging van MAbs-anti-afstotingstherapie aan de basis-immunosuppressie correleerde niet significant met het optreden van dood. Echter, het blijft zeer waarschijnlijk dat in directe samenhang met het aantal immunosuppressieve medicamenten en de duur van toediening, het optreden van neveneffecten wordt verhoogd[32].

Alhoewel onze populatie klein is, werd een verhoogde predispositie gevonden tussen de apen die MAbs-anti-afstotings therapie hadden ontvangen en ontwikkeling vertoonden van PTLP-afwijkingen ($P=0.03$, eenzijdige Fisher-test). De gebruikte MAbs waren specifiek gericht op lymfocyten, wat eerder het ontstaan van lymfoïde dan andersoortige maligne afwijkingen in de hand kan hebben gewerkt. Als alternatief moet worden opgemerkt dat virusinfecties, met name Epstein-Barr-[33] en Humaan T-Leukemie Virus (HTLV) -infecties, een mogelijk oorzakelijk verband hebben met de ontwikkeling van lymfoma's. Ogenschijnlijk waren slechts twee van de vier apen die PTLP-afwijking vertoonden seropostief voor antilichamen tegen Simiaan-TLV (STLV)[p] (ChIII,D). Echter, alle vier gevallen die PTLP-afwijkingen hadden ontwikkeld bleken de aanwezigheid van STLV-provirus op DNA-niveau in het lymfoproliferatieve weefsel te hebben (ChIII,E)[q]. Dit impliceert een mogelijke rol van STLV bij de ontwikkeling van maligne afwijkingen in combinatie met additionele immunosuppressie. De diversiteit van de morfologische en fenotypische PTLP-karakteristieken[r] duidt niet op een eenduidige oorzaak achter de

---

[p]simia = aap

[q]In alle gevallen bleek dus in het erfelijk materiaal van de lymfoïde tumor die van apen-origine was, erfelijk materiaal van het virus te zijn ingebouwd.

[r]de vorm en structuur (morfologie) van de tumor werd bepaald met algemene lichtmicroscopie op standaard gekleurde coupes; de verschijningsvorm (fenotype) van de tumor werd bepaald met immunohistochemische kleuringen.

ontwikkeling van deze afwijkingen.

Het lijkt duidelijk dat de beperkingen van de huidig gebruikte immunosuppressieve behandeling aangeven dat verder onderzoek op dit gebied geïndiceerd is voordat bij de mens de allogene hand getransplanteerd kan worden. Met dit idee werd het immunosuppressieve effect van twee nieuwe MAbs specifiek voor potente immunomodulatoire cytokines onderzocht in een huidentransplantatiestudie bij de rhesusaap (ChIV,A). Profylactische behandeling met alleen een anti-interferon-gamma of anti-tumor necrosis factor-alpha specifiek MAb, verlengde de huidoverlevingstijden niet. Neutralisatie van slechts één van beide lymfokines was blijkbaar niet afdoende om de immuunrespons te onderdrukken. Echter, behandeling met de combinatie van deze twee MAbs resulteerde in een bescheiden maar significante verlenging van de allotransplantaat-overlevingstijden. Vergeleken met MAbs, specifiek voor MHC klasse II en CD8-positieve cellen die respectievelijk een gemiddelde huidoverlevingstijd bewerkstelligden van 12.4 en 12.9 dagen[35] (ChII,D), was dit immunosuppressieve effect van de combinatie van anti-interferon-gamma en anti-tumor-necrosis factor-alpha even sterk.

Eveneens werd een huidtransplantatiestudie bij de rat verricht om het nieuwe medicijn FK-506 te testen (ChI,B.3.1.6 & ChIV,B). Toediening van 0.1 mg FK-506 voor de duur van 15 dagen had een gelijkwaardig effect op de huidtransplantaatoverleving als toediening van 5 mg CyA voor dezelfde duur. Bovendien werd in overeenkomst met andere experimentele data geconstateerd dat minimale therapeutische doseringen van FK-506 synergistisch reageerden met suboptimale doseringen CyA[36,37]. Kortgeleden bleek ook uit een klinisch onderzoek dat orale toediening van FK-506 in lage doseringen resulteerde in een effectieve en veilige immunosuppressie bij lever-, nier- en alvleesklier-transplantatie[38]. Naar aanleiding van deze gegevens lijkt het zinvol de immunosuppressieve potentie van FK-506 te onderzoeken in een allogeen samengesteld-weefsel transplantatiemodel. Voorlopige resultaten op dit gebied lijken veelbelovend[39].

Conclusies

Technische aspecten

Studies naar de anatomie van de hand van de rhesusaap kunnen ondersteunen dat bevindingen uit handtransplantatie-experimenten bij de rhesusaap geëxtrapoleerd kunnen worden naar de situatie bij de mens.

Technische haalbaarheid van het onderzoeksmodel kon aangetoond worden in de replantaties. Eveneens kon allogene transplantatie van de radiaire zijde van de hand technisch

gerealiseerd worden in het rhesusaapmodel.

De wondgenezing was ongestoord in zowel replantatie als transplantatie.
Postoperatieve bewakingssystemen zoals temperatuurmeting en laserdopplerflowmetrie hebben geen voorspellende waarde gehad voor vaatafsluiting noch voor begin van afstoting, doordat ze onder andere niet continu konden worden toegepast.

Functionele aspecten

De duim was weer volledig gereïntegreerd in de handfunctie na *autologe re*plantatie. Er trad een zeer goed sensibel en motorisch functioneel herstel op. Sensibel en motorisch herstel kon eveneens worden aangetoond na *allogene trans*plantatie van de radiaire zijde van de hand. Afstoting bewerkstelligde een daling in functioneel herstel, welke weer grotendeels herstelde na omkering van dit proces.

Evaluatie van sensibel en motorisch functieherstel kon slechts tot een half jaar postoperatief plaatsvinden. Desondanks bereikte het sensibele functieherstel in de niet afgestoten transplantaten waarden die de contralaterale zijde benaderden.

Ten aanzien van het motorisch herstel kan vermeld worden dat de waarden van zowel de amplitude van de samengestelde motor actiepotentialen van de thenarspieren, als de latentietijd van de distale thenar spier, gemeten door electromyografie, in de niet afgestoten transplantaten elkaar eveneens tegemoet kwamen.

Immunohistochemische kleuring liet vitale motor-eindplaatjes zien als bewijs voor motorische reïnervatie.

Immunologische aspecten

In huidtransplantatiestudies bij de rhesusaap werd de immunosuppressieve potentie getest van een combinatie van zeven monoclonale antilichamen (MAbs) specifiek voor de volgende cel-oppervlaktestructuren: CD3 (pan T-cel), CD4 (helper/inducer T cel-subset), CD8 (cytotoxische/suppressor T-cel subset). Tesamen bleken deze MAbs een additief effect te hebben bovenop hun individuele effect om de transplantaatoverleving te verlengen.

Kontinue hoge doseringen van cyclosporine A en prednison bewerkstelligden lange termijn overleving bij zes van de twaalf transplantaatontvangers. De hierboven genoemde MAbs-therapie kon afstoting omkeren en de transplantaatoverlevingstijden significant sterker verlengen dan anti-afstotingstherapie met steroïden. MAbs-therapie induceerde een sterke eliminatie van voornamelijk CD2+, CD3+ (=pan T-cel), en CD8+ (=cytotixische/ suppressor T-cel subset) lymfocyten. Eliminatie van deze witte bloedcelsubsets werd zowel gezien in het perifere bloed (mbv een immunofluorescentie-techniek) als in het allotransplantaat zelf (mbv immunohistochemische kleuringen).

Ondanks deze en bovengenoemde resultaten bracht het huidig gebruikte immunosuppressieve regime een aantal ernstige complicaties met zich mee: zeven rhesusapen overleden tijdens het experiment. In vier van deze gevallen traden posttransplantatie lymfoproliferatieve (PTLP)-afwijkingen op en in alle deze gevallen werd het STLV-provirus aangetoond in het DNA van deze afwijkingen. Het is dus zeer goed mogelijk dat STLV in combinatie met hooggedoseerde immunosuppressieve therapie een rol speelt bij het ontstaan van PTLP-afwijkingen. Het dient vervolgens opgemerkt te worden dat ook het toevoegen van de MAbs aan de basis-immunosuppressieve therapie (zwak) correleerde met het ontstaan van lymfoproliferatieve afwijkingen.

In het kader van de speurtocht naar immunosuppressiva om afstoting beter onder controle te houden en neveneffecten te minimaliseren werd een synergistisch effect gevonden tussen enerzijds MAbs specifiek voor de cytokines interferon-γ en tumor-necrosis-factor-α en anderzijds tussen cyclosporine A en het nieuwe immunosuppressieve middel FK-506.

Toekomstverwachtingen

Allogene transplantatie van de allogene hand lijkt gezien de veelbelovende resultaten op technisch, functioneel en immunologisch gebied dichter bij de realiteit zijn komen te staan. Echter, het feit dat met de hoge doseringen van de beste immunosuppressieve middelen hedentendage beschikbaar nog steeds zeven van de twaalf rhesusapen voortijdig overlijden, maakt het, naar onze mening, noodzakelijk te zoeken naar een effectiever minder toxisch immunosuppressieve therapie voordat (totale) handtransplantatie bij de mens verricht kan worden uitgevoerd.

Indien eenmaal een dergelijk middel of combinatie van middelen beschikbaar is gekomen, dan zal zeer wel mogelijk sprake zijn van een indicatiegebied voor functionele en esthetische reconstructie met gebruikmaking van allogene weefsels.

Referenties

1.   Merion RM, White DJG, Thiru S, et al. Cyclosporine: Five years' experience in cadaveric renal transplantation. N Eng J Med 1984; 310: 148.
2.   Hors J, Busson M, Bouteiller AM, Raffoux C, Betuel H, Fauchet R, Tongio MM, Mercier P, Lepetit JC, Dausset J: Dissection of the respective importance of HLA-A,B,DR matching in 3,789 prospective kidney transplants. Transpl Proc 1987; 19: 687.
3.   McGregor CGA, Oyer PE, Shumway NE. Heart and heart-lung transplantation. Prog Allergy 1986; 38: 346.
4.   Starzl TE, Iwatsuki S, Shaw BW Jr, et al. Orthotopic liver transplantation in 1984. Transplant Proc 1985; 17: 250.

5. Ringden O. Cyclosporine in allogeneic bone marrow transplantation. Transplantation 1986; 42: 445.

6. Brooks JR: Where are we with pancreas transplantation? Surgery 1989; 106: 935.

7. Watson AJ, Lear PA: Current status of intestine transplantation. Gut 1989; 30: 1771.

8. Ding YL, Han CM: Recent advances in burn wound management in China. Actua Chir Plast 1989; 31: 84.

9. Schwind JV: Homotransplantation of extremities of rats. Radiology 1962; 78: 806-809.

10. Doi K: Homotransplantation of limbs in rats: a preliminary report on an experimental study with nonspecific immunosuppressive drugs. Plast Reconstr Surg 1979; 64: 613-624.

11. Black KS, Hewitt CW, Fraser LA, et al: Cosmas and Damian in the laboratory. N Eng J Med 1982; 306: 368-369.

12. Furnas DW, Black KS, Hewitt CW, et al: Indefinite composite tissue (limb) allograft survival in rats with cyclosporine : Possible furure implications in plastic and reconstructive surgery. Transpl Proc 1983; 15: 3063.

13. Hewitt CW, Black KS, Fraser LA, et al: Cyclosporine A (CyA) is superior to prior donor-specific blood (DSB) transfusion for the extensive prolongation of rat limb allograft survival. Transpl Proc 1983; 15: 514-517.

14. Fritz WD, Swartz WM, Rose S, Futrell JW, Klein E: Limb allografts in rats immunosuppressed with cyclosporine A. Ann Surg 1984; 199: 211.

15. Black KS, Hewitt CW, Fraser LA, et al: Composite tissue (limb) allografts in rats. II. Indefinite survival using low-dose cyclosporine. Transplantation 1985; 39: 365.

16. Hewitt CW, Black KS, Dowdy SF, et al: Composite tissue (limb) allografts in rats. III. Development of donor-host lymphoid chimeras in long-term survivors. Transplantation 1986; 41: 39.

17. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21: 3191.

18. Jonker M, Goldstein G, Balner H: Effects of in vivo administration of monoclonal antibodies specific for human T cell subpopulatioons on the immune system in a rhesus monkey model. Transplantation 1983; 35: 521.

19. Jonker M: The importance of nonhuman primates for preclinical testing of immunosuppressive monoclonal antibodies. Seminars in Immunology, 1990, (in press).

20. Daniel RK, Egerszegi EP, Samulack DD, Skanes SE, Dykes RW, Rennie WRJ: Tissue transplants in primates for upper extremity reconstruction: a preliminary report. J Hand Surg 1986; 11A:1.

21. Stark GB, Swartz WM, Narayanan K, Moller AR: Hand transplantation in baboons. Transplant Proc 1987; 19: 3968.

22. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. New Eng J Med 1985; 313 (6): 337.

23. Ponticelli C, Rivolta E, Tarantino A, Egidi F, Banfi G, DeVecchi A, Montagnino G, Vegeto A: Clinical experience with ORTHOCLONE OKT3 in renal transplantation. Transpl Proc 1986: 18: 942.

24. Starzl TE, Fung JJ. ORTHOCLONE OKT3 in treatment of allografts rejected under Cyclosporine-steroid therapy. Transpl Proc 1986; 18: 937.

25. Hovius SER, Stevens HPJD, Jonker M, van der Meulen JC: Partial hand transplantation in the rhesus monkey: Phase I: Technical feasibility of the model. Annual Report REP-TNO, 1987: 197.

26. Armitage P, Berry G. Factorial design. In: Armitage P eds. Statistical methods in medical research.

London, UK: Blackwell Scientific Publications, 1987: 227.

27. Skanes SE, Samulack DD and Daniel RK. Tissue transplantation for reconstructive surgery. Transpl Proc 1986; 18(4): 898.

28. Vreeswijk W, Balner H. Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys. Transplantation 1980; 30 (3): 196.

29. Hewitt CW, Black KS, Stenger JA, Go C, Achauer BM, Martin DC. Comparison of kidney, composite tissue and skin allograft survival in rats prolonged by donor blood and concomitant limited cyclosporine. Transpl Proc 1988; 20 (3,S3): 1110.

30. Lagaaij EL, Henneman IPH, Ruigrok M, etal: Effect of one-HLA-DR-antigen-matched and completely HLA-DR-mismatched blood transfusions on survival of heart and kidney allografts. N Engl J Med 1989; 321: 701.

31. Samulack DD, Munger BL, Dykes RW, Daniel RK: Neuroanatomical avidence of reinnervation in primate allogarfted (transplanted) skin during cyclosporine immunosuppression. Neurosci Lett 1986; 72: 1.

32. Scott JP. Higenbottam TW. Med Toxicol 1988; 3: 107.

33. Penn I: Cancers following cyclosporine therapy. Transplantation 1987; 43: 32.

34. Hanto DW, Gajl-Peczalska KJ, Frizzera G, etal: Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occuring after renal transplantation. Clinical, pathological, and virological findings and implications for therapy. Ann Surg 1983; 198: 356.

35. Clark JW, Robert-Guroff M, Ikehera O, Henzen E, Blattner WA: Human T-cell leukemia-lymphoma virus type 1 and adult T-cell leukemia-lymphoma in Okinawa. Cancer Res 1985; 45: 2849.

36. Jonker M, Nooij FJM, Suylichem P van, Neuhaus P, Goldstein G. The influence of OKT8F treatment on allograft survival in rhesus monkeys. Transplantation 1986; 41(4): 431.

37. Murase N, Todo S, Lee PH et al. Transplant Proc 19:71,1987

38. Zeevi A, Duquesnoy R, Eiras G et al.: Transplant Proc 20:220, 1988

39. Starzl TE, Todo S, Fung J, etal: FK-506 for liver, kidney and pancreas transplantation. Lancet 1989; 2: 1000.

40. Arai K, Hotokebuchi T, Miyahara H, Arita C, Mohtai M, Sugioka Y, Kaibara N: Prolonged limb allograft survival with short-term treatment with FK-506 in rats. Transpl Proc 1989; 21(1): 3191-3193.

ACKNOWLEDGEMENTS

CURRICULA VITAE

Steven Hovius was born on June 1st, 1951 in Punta Cardon, Venezuela. After graduating from secondary school (HBS-B, Coomhert Lyceum in Haarlem) in 1968, he studied at the Medical Faculty of the 'Vrije Universiteit' in Amsterdam until 1977.

After military service in the Royal Dutch Navy he started his training in general surgery in 1978 in the Academic Hospital 'Binnengasthuis' in Amsterdam (Head: Prof. Dr W.H. Brummelkamp). He was registered as general surgeon in 1984. From 1984 to 1987 he was trained as a plastic surgeon at the Academic Hospital 'Dijkzigt' in Rotterdam (Head: Prof. Dr J.C. van der Meulen).

The experimental work with the rhesus monkeys was started in 1987 at the Primate Center TNO, Rijswijk, and performed under the supervision of Dr M. Jonker.

Since his registration as a plastic surgeon on January 1st 1987 he has been working as a 'chef de clinique' in the same department.

Jeroen Stevens was born on December 6th, 1962 in The Hague, The Netherlands. He attended gymnasium ß at the 'Stedelijk Gymnasium' in Breda, where he graduated in 1981, cum laude. Medical studies were started at the State University Antwerp, Belgium. After completion of his first year (cum laude), he continued his studies at the Erasmus University in Rotterdam (EUR) in 1982. He qualified for the degree of Master in Science in January 1987.

From 1983 to 1986 he was a student-assistant at the department of Neuroanatomy, EUR (Head: Prof. Dr H.G.J.M. Kuypers). After a student-assistancy from 1985-1986 at the department of Plastic Surgery, University Hospital 'Dijkzigt', Rotterdam (Head: Prof. Dr J.C. van der Meulen) he was a research fellow at the same department from 1986 till 1991. The experimental work with the rhesus monkeys was started in 1987 at the Primate Center TNO, Rijswijk, and performed under the supervision of Dr M. Jonker.

Presently, he is completing his clinical clerkships to finish his medical studies.

PUBLICATIONS USED FOR THIS DOUBLE-THESIS

The thesis of S.E.R. Hovius is based on the following publications*:

Hovius SER, Adrichem LNA van, Heijden PMA van der, Vuzevski VD, Strik R van, Meulen JC van der: Postoperative monitoring of allogeneic limb transplantation in rats. *Ann of Plast Surg 1988; 21(6): 559-565.*

Hovius SER, Stevens HPJD, Peters B, Nierop PWM van: A protective thermoplast splint for the forearm and hand in nonhuman primates. *Lab Animals, 1991; 25: 1-3.*

Hovius SER, Stevens HPJD, Nierop PWM van, Rating W, Strik R van, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. I. Technical aspects. *J Plast Recon Surg (accepted for publication).*

Hovius SER, Stevens HPJD, Meulen JC van der: Replantation of the radial side of the hand: anatomical and functional aspects of a research model *Br J Hand Surg (accepted for publication).*

Hovius SER, Nierop PWM van, Stevens HPJD, Godschalk M, Kusuma A, Mechelse K, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. II. Functional aspects. *J Plast Recon Surg (accepted for publication).*

Stevens HPJD, Hovius SER, Nierop PWM van, Rating W, Kwast ThH van der, Jonker M, Meulen JC van der: Immunological aspects of partial hand transplantation in the rhesus monkey. *Transpl Proc 1990; 22: 2006-2008.*

Stevens HPJD, Hovius SER, Heeney JL, Jonker M: Immunological aspects and complications of composite tissue allografts for upper extremity reconstruction in rhesus monkeys. *Transpl Proc 1991; 23(1): 623-625.*

Stevens HPJD, Hovius SER, Kwast ThH van der, VD Vuzevski, Götte M, Roche NA, Jonker M: Composite tissue allografting: Effects of cyclosporine A, prednisone, blood transfusions and monoclonal antibodies. *Transplantation (submitted).*

Dammeijer PFM, Stevens HPJD, Hovius SER, Marquet RL: Combined effects of low-dose FK-506 and Cyclosporine A on skin graft survival. *Transpl Proc 1990; 22: 1653-1654.*

Stevens HPJD, Roche NA, Vuzevski VD, Hovius SER, Jonker M: In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive antigens. *Transpl Proc 1990; 22: 1783-1784.*

The thesis of H.P.J.D. Stevens is based on the following publications*:

Stevens HPJD, Roche NA, Vuzevski VD, Hovius SER, Jonker M: In vivo immunosuppressive effects of monoclonal antibodies specific for CD3+, CD4+, CD8+ and MHC class II positive antigens. *Transpl Proc 1990; 22: 1783-1784.*

Stevens HPJD, Kwast ThH van der, Buurman W, Meide PH van der, Jonker M: In vivo synergistic effects of monoclonal antibodies specific for interferon gamma and tumor necrosis factor alpha. *Transpl Proc 1990; 22: 1924-1925.*

Stevens HPJD, Kwast ThH van der, Meide PH van der, Vuzevski VD, Buurman W, Jonker M: Synergistic immunosuppressive effects of monoclonal antibodies specific for interferon gamma and tumor necrosis factor alpha. *Transplantation 1990; 50(5): 856-861.*

Stevens HPJD, Hovius SER, Nierop PWM van, Rating W, Kwast ThH van der, Jonker M, Meulen JC van der: Immunological aspects of partial hand transplantation in the rhesus monkey. *Transpl Proc 1990; 22: 2006-2008.*

Stevens HPJD, Hovius SER, Heeney JL, Jonker M: Immunological aspects and complications of composite tissue allografts for upper extremity reconstruction in rhesus monkeys. *Transpl Proc 1991; 23(1): 623-625.*

Stevens HPJD, Hovius SER, Kwast ThH van der, VD Vuzevski, Götte M, Roche NA, Jonker M: Composite tissue allografting: Effects of cyclosporine A, prednisone, blood transfusions and monoclonal antibodies. *Transplantation (submitted).*

Stevens HPJD, Kwast ThH van der, Timmermans A, Stouten N, Jonker M: Monoclonal antibodies for immunohistochemical labelling of immunocompetent cells in frozen sections of rhesus monkey tissues. *J Med Prim (submitted).*

Stevens HPJD, Hotterman L, Haaksma AGM, Goudsmit J, Heeney JL: Development of STLV-provirus positive lymphoid tumors in rhesus monkeys immunosuppressed for transplantation. *Lab Invest (in preparation).*

Dammeijer PFM, Stevens HPJD, Hovius SER, Marquet RL: Combined effects of low-dose FK-506 and Cyclosporine A on skin graft survival. *Transpl Proc 1990; 22: 1653-1654.*

Hovius SER, Stevens HPJD, Peters B, Nierop PWM van: A protective thermoplast splint for the forearm and hand in nonhuman primates. *Lab Animals, 1991; 25: 1-3.*

Hovius SER, Stevens HPJD, Nierop PWM van, Rating W, Strik R van, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. I. Technical aspects. *J Plast Recon Surg (accepted for publication).*

Hovius SER, Stevens HPJD, Meulen JC van der: Replantation of the radial side of the hand: anatomical and functional aspects of a research model *Br J Hand Surg (accepted for publication).*

Hovius SER, Nierop PWM van, Stevens HPJD, Godschalk M, Kusuma A, Mechelse K, Meulen JC van der: Allogeneic transplantation of the radial side of the hand in the rhesus monkey. II. Functional aspects. *J Plast Recon Surg (accepted for publication).*

*Both authors were permitted to present all publications that are copyright-protected in this double-thesis.