A STRATEGY FOR PERIPHERAL NERVE ALLOGRAFTING: IMMUNOSUPPRESSION VERSUS CHIMERIC NERVE GRAFTING

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SUMMARY

Chimera: (Biology) An organism consisting of at least two genetically different kinds of tissue as a result of mutation, grafting, etc.
Collins English Dictionary

This thesis has set out to investigate the regenerative potential of peripheral nerve allografting in a rodent model. In particular, the strategy of reconstructing a nerve defect using a novel chimeric graft of mixed antigenicity (*Sandwich graft*) was explored as an alternative to autografting or immunosuppression.

Peripheral nerve injury produces significant individual and community morbidity. Despite technical and surgical advances, even the results from urgent repair of single modality nerves are sub-optimal. This situation is compounded when nerve structure is lost and a bridging conduit is required to reconstruct the defect. While many organic and inorganic materials have been investigated, autografting remains the mainstay of current clinical practice. However, suitable autografts are in limited supply and associated with donor site morbidity.

Like autografts, peripheral nerve allografts (PNAG) possess all the features required of an ideal nerve conduit, especially Schwann cells. Studies with acellular conduits have shown the addition of Schwann cells is advantageous to regeneration. However, immunological rejection of antigenic Schwann cells within nerve allografts renders the graft acellular, inhibiting regeneration. Immunosuppression preserves allogenic Schwann cells and results in regeneration equivalent to autografts. But immunosuppressants are associated with serious side effects such that their use in peripheral nerve injuries is deemed unethical.

Temporary immunosuppression has been proposed as a solution to this problem until the non-antigenic host axon regeneration is established. However this has produced conflicting results although an axonopathic process following drug withdrawal is acknowledged.

The aims of this project were firstly to assess the regenerative potential of non-immunosuppressed PNAGs. The hypothesis being that rejection would produce acellular grafts as donor Schwann cells were destroyed. Host Schwann cells are known to repopulate acellular conduits from adjacent nerve ends. By using a chimeric Sandwich graft, the second aim of the project was to assess whether augmenting host Schwann cell graft repopulation could improve axonal regeneration and reduce immunosuppressant requirements.

A clinically reproducible chimeric construct of a nerve allograft with an intervening segment of autologous nerve (a *Sandwich graft*) was used to bridge a standard traumatic nerve gap within the experimental groups. Comparisons were made between simple and sandwich grafts composed of autologous, isogeneic and allogenic nerves split into immunosuppressed and non-immunosuppressed limbs. Incompatible rat strains were employed using Cyclosporin (CyA) as the sole immunosuppressant. Qualitative and quantitative analyses of immunohistochemical and conventional histological specimens, along with measurement of Gastrocnemius muscle mass, were used to assess the outcome of nerve regeneration.

Analyses were undertaken within the early and late regeneration periods of 21 days and 32 weeks respectively. Immunohistochemistry and conventional histological techniques were employed, along with measurements of muscle mass recovery to indicate target organ reinnervation. Cyclosporin (CyA) was the sole immunosuppressants used.

Non-immunosuppressed PNAGs were shown to support less early axonal regeneration than their immunosuppressed counterparts and non-allogenic controls. This was associated with a reduction in Schwann cells and increased inflammation indicating rejection. However, the pattern of regeneration followed that of the other groups and improved with time. Sandwich allografting was associated with improved axonal regeneration, increased Schwann cells and less inflammation suggesting reduced antigenicity of the sandwich construct. The increased neurorrhaphies compared to simple grafts did not appear deleterious.

Long-term myelinated nerve counts and target organ reinnervation produced similar results from all experimental groups regardless of immunosuppression. This work validates the use of a chimeric sandwich graft within this model. The addition of a depot of host Schwann cells within an allograft without immunosuppression produces similar regeneration to non-allogenic grafts and reduces the early delay period associated with rejection.

Further analysis of the formulations of CyA used indicated that although oily and parenteral CyA did adequately immunosuppress the allografts, the results from parenteral CyA were marginally better. Also, CyA use did contribute to model morbidity and mortality within these experiments.

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AUTHOR'S DECLARATION

In keeping with the regulations for submission, I hereby declare that this thesis was composed by myself, and that unless otherwise stated, the work presented is my own.

All procedures involved in the experiments undertaken for this thesis were carried out by the author with the exception of the following:

1. Cyclosporin radioimmunoassay was undertaken by Dr. Mark Dunsford in the Biochemistry Dept. of Guy's Hospital, London.

2. Fixation of distal nerve segments for semithin analysis were carried out by Dr. Padmini Sarathchandra from the Pathology Department of Northwick Park Hospital, London.

Signed_____ (Fiona J. Hogg) Date_____

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Alk phos	alkaline phosphatase
ALT	alanine transaminase
ANOVA	analysis of variance
APC(s)	antigen presenting cell(s)
AST	aspartate aminotransferase
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
CD4+	T helper cells (Th) – subset of T lymphocytes
CD8+	cytotoxic T cells - subset of T lymphocytes
^{51}Cr	chromium 51 - radioisotope
Creat	creatinine
CyA	cyclosporin: an immunosuppressant agent
oCyA	oily cyclosporin solution prepared from CyA base powder
pCyA	parenteral formulation cyclosporin
CVA	cerebrovascular accident – "stroke"
DA	Dark Agouti: a stain of rats
DN	distal neurorrhaphy
DRG	dorsal root ganglion
ED1	a specific marker for rat macrophages
FITC	fluoroscein isothiocyanate
FK506	tacrolimus: an immunosuppressant agent
FPIA	fluorescence polarization immunoassay
GA	general anaesthesia
IFNγ	interferon gamma
IL-1	interleukin-1
IL-2	interleukin-2

Lew	Lewis: a stain of rats
MHC	major histocompatibility complex
NGF	nerve growth factor
NT3	neurotrophin-3
NT4/5	neurotrophin-4/5
OCT	Optimum Cutting Temperature compound
PamNF	panaxonal marker of neurofilaments
PAN/PVC	acrylonitrile vinylchloride
PBS	phosphate buffered saline
PHB	polyhydroxybutyrate
PLC	poly (L-lactide-ε-caprolactone) copolymer
PN	proximal neurorrhaphy
PNAG(s)	peripheral nerve allografts(s)
PTFE	polytetrafluroethylene
S100	a specific Schwann cell cytoplasmic antigen
SD	standard deviation
TNFβ	tumour necrosis factor beta
UV	ultraviolet
UWCSS	University of Wisconsin cold storage solution
VWF	von Willebrand factor, a specific endothelial cell surface antigen

CHAPTER ONE

Introduction

1.1 INTRODUCTION

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1.1 INTRODUCTION

A significant proportion of the reconstructive surgeon's workload involves dealing with trauma. According to The British Association of Plastic Surgeons, trauma comprises a third of their members' cases, with upper limb trauma forming the largest proportion of this group within Plastic Surgery practice [1] . Within this group there exists a population of patients who have sustained nerve injuries, either in isolation, or as part of a more complex destructive picture. With the addition of nerve injuries resulting from trauma to other body parts, whether accidental or as part of planned surgical resections, a sizeable source of community morbidity is identified.

The average number of patients with digital nerve injuries attending a Plastic Surgery unit serving a population of 1.2 million is between two and three hundred per year (Personal audit). Economically this group require specialist treatment for their condition and rehabilitation. Some may be permanently disabled and unable to work. Pathological conditions affecting the peripheral nervous system (as opposed to central and generalised conditions) are rarely life threatening, but are certainly life altering.

The peripheral nerve is a recognised anatomical structure whose physical integrity can be repaired, but unlike other organic conduit structures, the repaired nerve may not function normally afterwards despite a surgically accurate repair. Neurones are known to be sensitive to even minor degrees of assault [2,3] . The most minor type of injury results in neuropraxia, or localised conduction block without any break in the nerve structure [2,3] . This is the only type of injury where normal recovery of function is seen. Why this should be has been the focus of much research [4] .

Since the earliest recordings of written medicine, when Galen (130-201AD) recognised nerves as distinct anatomical structures, and not variants of tendons, their susceptibility to injury and loss of function has been noted. Early physicians also believed that divided nerves were incapable of uniting, and nerve injury and manipulation produced convulsions [5] . Historical reports of attempts to repair nerves identify that suturing and agglutination were sporadically favoured [6] . Paulus Aeginata is credited with the first recorded suture coaptation, in combination with agglutination in the 7th century [7] . Arabic physicians attempted nerve repairs by suturing in the 9th and 10th centuries [8] , while Roger of Parma in the 13th century tried egg albumin [6] . The reports of Guy de Chauliac in the 14th century observed that in cases of nerve suture in young patients: "afterwards one could not believe that they had been cut" [9] . The superior regenerative potential of the young is recognised today. However for most of the last millenium there has been little progress in the repair of nerve injuries. Even up to the 19th century it was commonly believed that manipulation of severed nerve ends led to convulsions such that repair was attempted by approximation of surrounding tissues (repair cum carne) [10] . By the end of the 19th century a perceived increase of interest in nerve pathophyiology is evident from the available medical literature on the subject. The first description of repair by epineurial suture is attributed to Hueter in 1873 [8,10] . This technique, although refined, is still practiced today. Some of the reasons for poor results from suturing in the past are recognised now as scarring at the coaptations, reactions to sutures and foreign bodies, infection and complication from poor surgical technique. Modern developments have led to improvements in some of these areas, however in their book of 1994, MacKinnon and Dellon reported that even with digital nerve repairs in the post-microsurgical era, 89% of patients reported abnormal return of sensation. With more proximal injuries sensation and motor function showed more imperfect return [11] . While medical literature is littered by reports of successful interventions improving nerve regeneration, the stark truth is that none produce results approaching the functional capabilities of intact nerve, including distal injuries in single modality nerves [4,11] .

1.2 NERVE INJURY AND REGENERATION

Following nerve injury, a process of regeneration and repair is initiated. Unless the injury is of a Sunderland Grade I [2,3] , all other degrees of damage require repair processes involving the many cell types which make-up an anatomical peripheral nerve. The most serious injury is where nerve physical continuity is broken, resulting in a simple laceration or loss of a portion of nerve substance. However, many injuries show a mixed picture of nerve structural damage.

The structural elements of peripheral nerves include the neuronal axons, Schwann cells and their myelin sheaths, connective tissues forming the epi-, peri- and endoneurium, blood vessels, lymphatics and immunocompetent cells. All of these elements function in concert but the relative importance of each may change after injury and these variations can have positive and negative effects on the regenerative processes.

1.2.1 Neuronal response to injury

With nerve transection, conduction is interrupted. The cell body exhibits chromatolysis indicating a switch of metabolic function from transmission to one of manufacture, with an increase in the synthesis of regeneration associated proteins such as tubulin and actin to facilitate regeneration and repair [12-15] . However, not all neurons effect this transition. Significant numbers of neurons undergo cell death which has been reported to between 20 - 50% in studies of dorsal root ganglia (DRG) neurons post sciatic nerve transection [4,16-18] . Extrapolation of this fact indicates that post regeneration function is never going to be the same as normal nerve until the mechanisms surrounding apoptosis are identified and reversed.

Following nerve injury, the severed ends retract exposing vascular endothelium which stimulates coagulation with reduction of haemorrhage and relative ischaemia

at both cut nerve ends. Endothelial exposure, along with axon exposure and axoplasm leak, compounded by recoil of the perineurial connective tissues results in fusing of the axonal membranes along with initiation of the acute inflammatory response [19] . Local oedema results in swelling and compounds local ischaemia. Destruction by free radicals is facilitated by calcium ion influx into the axoplasm which activates the necessary proteases [4,20,21] . The active production of cytokines by the intrinsic cell population signals further influx of circulating immunocompetent cells and amplification of the cascade reactions in progress [22-24] . Macrophage recruitment is essential to effective Wallerian degeneration [12,25-27] , with degradation of the distal axon resulting from release of myelinolytic neutral proteases [28] .

The distal nerve, and proximal nerve stump up to the last Node of Ranvier undergo Wallerian degeneration peaking at 3 days after injury and lasting for approximately 2 weeks [29,30] . Myelin is degraded after separation from the endoneurial tube and distal axon and phagocytosed along with axonal debris by macrophages and Schwann cells [4,14,31,32] . Wallerian degeneration is also associated with Schwann cell proliferation which leaves this population aligned within the endoneurial tubes as bands of Büngner awaiting the regenerating axonal process [14,31-33] . The endoneurial tubes reduce their volume following clearance of axoplasm and myelin, and despite Schwann cell proliferation these tubes shrink if not reinnervated, narrowing by 80-90%, although remaining patent for some years [34,35] . Deposition of new endoneurial collagen also affects the ability of the endoneurial tube to expand when reinnervated. These two factors result in axons of smaller diameter than in the uninjured nerve which affects conduction properties and function following regeneration. The parent axon and Schwann cells also influence this outcome [14,36] .

1.2.2 Axonal Regeneration

The axon regenerates by producing multiple sprouts [9,13,20] . This is initiated within hours of the injury and established by 27 hours, although the controlling mechanisms are as yet unknown. Specialised growth cones [30,37] are found at the tip of each sprout and support multiple motile filopodia [4,31] , which respond to contact guidance cues [38,39] . Cell adhesion molecules expressed by growth cones and Schwann cells are also important in the regeneration process [36-40] . The neuron manufactures cell membrane proteins which axoplasmic flow delivers to the advancing growth cone and site of membrane addition [14,26,31] . Axonal regeneration requires a suitable matrix or substrate, direction from guidance cues and the presence of trophic substances to support and direct regeneration. Schwann cells have been found to contribute in all these areas. Although the regeneration process is similar between species, these have different nerve regeneration rates, being 1-2mm per day in humans [36,41] , and up to 3.5mm per day in rodents. Regeneration rate is also affected by scarring, distance from the cell body and age [36,42] .

Maturation of the axon continues behind the advancing growth cone with myelination of fibres and increasing axonal girth. The myelin sheath thickens with time, while non-myelinating axons form small groups within the folds of individual Schwann cells. With completion of end organ connections, a delay follows allowing functional maturation before conduction can be re-established [43,44] .

1.3 FACTORS AFFECTING REGENERATION

Rarely, injuries are simple and although regeneration has been established there are many variables which may affect the progress and result of nerve regeneration.

Topographical orientation of two severed nerve ends is difficult to align accurately, especially if tissue has been lost. Axons are impossible to align with current technology, and made more difficult because of the plexus arrangement of axons within nerves [2] . Also minimising surgical manipulation and thorough debridement of damaged tissue are all techniques known to minimise scarring in nerves as in other tissues [45] .

The development of microsurgery, with its specialised instrumentation and magnification, has been paramount in improving the quality of neurorrhaphy attainable [46,47] . However, it is obvious that a plateau has been reached with this technology [4,12] while the ultimate aim of perfect nerve regeneration remains an unfulfilled goal.

The quality of the surgical repair certainly has a profound effect on the eventual outcome, possibly more important here than in other areas of reconstructive surgery [11] . However as exact fascicle to fascicle coaptations are difficult, and certainly endoneurial tube to endoneurial tube coaptations are beyond the scope of current human endeavor other strategies have been assessed to improve regeneration.

Forssman suggested the concept of neurotrophism in1898 [48] , while Cajal in the 1920's suggested a neurotrophic influence played a part in the specificity of axonal regeneration [30] . The first neurotrophic factor to be isolated and defined in 1953 was nerve growth factor (NGF) [49] . Since then other neurotrophic factors such as neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and brain derived neurotrophic factor (BDNF) and many more have been identified [14,31,48] . With each, their varying actions in growth, regeneration and degeneration of the nervous system are being studied [50,51] .

1.4 NERVE GAPS AND CONDUITS

Nerve injury resulting in the loss of continuity of the structure can be repaired by a single coaptation. The problems of a single neurorrhaphy are multiplied when a portion of nerve is lost, and the gap has to be repaired using material which can support axonal regeneration.

The ideal conduit material should be readily available, biocompatible, biodegradable, supportive and protective of regeneration while also exhibiting good handling characteristics and be reproduceable in a variety of dimensions inexpensively. An internal structure to support regeneration is a desirable quality, as it would guide and improve regeneration.

Conduit materials can either be organic or inorganic. With increasing technology successive generations of scientists and surgeons have utilised and manipulated every conceivable material as potential nerve conduits [52] . Lundborg has used silicone tubes clinically [53,54] and also has found, along with other researchers, that the tube concept is also useful for studying regeneration and its influences [4,52,55,56] . However, solid tubes block blood vessel in growth from the wound bed therefore they are only useful over short distances [57] . Permeable conduit materials and meshes go some way to addressing these problems and are generally associated with improved regeneration [52] . Synthetic tubes manufactured from biodegradable or organic components show more promise of fulfilling the criteria of the ideal conduit material. Some like PTFE (polytetrafluroethylene), polyglycolic acid [58-60] , PAN/PVC (acrylonitrile vinylchloride) [61] , and PLC (poly (L-lactide-ε-caprolactone) copolymer [62] form hollow tubes, while others like fibronectin [63-65] produce a mesh. Another synthetic conduit of interest is PHB (Polyhydroxybutyrate). Initial studies in animals [60,66] have had sufficient beneficial results to initiate a clinical trial which is currently in progress.

Non-neuronal glial cells migrate into the conduit material from both cut nerve ends supporting axon regeneration [52,63,67] . This is therefore the limiting event as without the correct cell population and mix of factors provided by these cells, axonal development will be limited.

Organic materials have also been used as nerve conduits, including bone, arteries [52] , veins [68,69] , fallopian tube [70] and muscle [71-73] . The findings of cellular migration into acellular conduits of any type bring about the conclusion that Schwann cells are an essential component for axonal regeneration [52,74-77] . For this reason, (as well as providing a basal lamina scaffold) peripheral nerve grafts are used as the standard material to repair nerve gaps clinically, providing the most successful conduit material for the reconstruction of peripheral nerves [4,36] . Nerve autografts are the gold standard against which all other conduits and methods to repair nerve gaps are measured [52,78] .

1.5 NERVE AUTOGRAFTS

Interest in peripheral nerve autografting followed the clinical case reported by Philipeaux and Vulpian in 1870. This case of successful regeneration following lingual nerve repair using a graft of hypoglassal nerve in a dog is the first attributed to the "modern" era of medical history [79] . Following on from both World Wars in the 20th century there was increasing interest in all aspects of nerve injury, repair and grafting [80-82] . The increased use of nerve grafts during World War II, the general concensus was that clinical results were poor [83,84] . Attempts were made to improve the outcome of autografting with Seddon and Sanders in the 1940s advocated the use of cabled nerve grafts to avoid the central necrosis observed in trunk nerve grafts [81,85] . However, despite extensive reviews of experience over this period the technique remained peripheral in the management of nerve injuries [45] . Since then improvements in surgical technique and magnification, along with

greater understanding of graft physiology and non physiological tension across the repairs have improved the results from grafting [3,11,46-48,86-88] .

Studies on nerve autografts have confirmed that the structure and the presence of Schwann cells are required for optimal axonal regeneration across the graft [20,34,52,75,78,89,90] . The graft provides an ideal structure for axonal regeneration with a basal lamina scaffold and extracellular matrix for contact guidance [4,12,31,91] . Schwann cells supply neurotrophic factors [33,75,92,93] and along with macrophages [19,25,27,94] prepare the endoneurial tubes for reinnervation, while cell adhesion molecules on the basal lamina and perineural cell surfaces aid axonal elongation [38,39] . However, the main problem associated with nerve autografts is donor site morbidity. There is also a finite limit to the amount of nerve any patient or surgeon is prepared to sacrifice to reconstruct a damaged nerve, especially when the results are likely to be suboptimal [42,78] .

1.6 GRAFTING ACROSS IMMUNOLOGICAL BARRIERS

Interest in nerve allografting and xenografting has mirrored the historical developments of nerve autografting. The high demand for nerve reconstruction during periods of conflict, and the obvious limits of autograft supply fuelled this demand.

1.6.1 Xenografts

Ten years following Philipeaux and Vulpian's autograft report, in 1880, Glück claimed success following a xenograft of 3cm fowl nerve to rabbit [95] . Follow up consisted of a pinch test resulting in a distal muscle twitch at 11 days. A repeat of the experiment by Johnson in 1919 showed that while the xenograft had joined with the donor, the nerve did not function at 23 days [96] . This identifies a problem with historical reporting of nerve repair techniques in that more emphasis was

placed on whether the nerve had healed than on the functional outcome [97,98] . There was often no control limb to the experiments and variable standards of reporting.

The two world wars stimulated research into many experimental nerve conduits including encouraging previously discouraged investigators to continue xenografting. Early attempts to reduce the host response to the xenograft included fixation in alcohol storage in physiological saline and petroleum [97] . These methods predated widespread knowledge of immunology, therefore it is not surprising that xenografts failed.

Sanders and Young in 1942 silenced xenograft research for decades when their experiments found no value in fresh, stored or alcohol fixed nerve xenografts. They concluded that xenografts were treated as foreign bodies by the host and were ulitmately replaced [85] . Marmor resurrected the xenograft debate in the 1960s, reporting variable results in dog, rat and guinea pig models using xenografts pretreated by freezing and irradiation sometimes in combination with silastin or vascular cuffs. Variable success was also noted using azathioprine immunosuppression [99,100] . However the poor results achieved by these pretreatment methods in nerve allografts [101] contributed to the halting of nerve xenograft work except in regard to more basic areas such as Schwann cell and myelin responses [102,103] .

The experience with nerve xenografting should not be viewed in isolation. Throughout the 1960's in the USA clinical solid organ xenografts were courting controversy in the full glare of a global media. Although not mentioned in the scientific literature, the reported cost both professionally and personally to the surgeons involved in these cases [104] must have influenced research in nerve xenotransplantion. Nerve xenotransplantation, after all was not being advocated in

pathological states where life was at risk to balance the ethical and moral dilemmas which were the Pandora's box of xenotransplantation then and now.

Recent advances in genetics have reopened the xenotransplantation debate as a potential source of donor organs [105] . David White with Imutran and Alexion Pharmaceuticals in the USA are pioneering work with transgenic pigs to block the complement cascade [104,106] . No work has yet been undertaken with transgenic nerve transplants.

1.6.2 Allografts

Interest in nerve allografts has as long a history as nerve conduits and stems from the same reasons: significant loss of structure requiring reconstruction to restore continuity. Nerve allografts seem a logical step from nerve autografts in humans, where anatomical nerves could be replaced like for like.

Albert is credited with the first human fresh nerve allograft in 1885. He harvested nerves from an amputated lower limb to repair median and ulnar nerves in two patients [107] . Follow up was limited but Huber reported that one graft was necrotic and removed at 6 days [98] . Mayo-Robson in1889 claimed success in one patient who had achieved normal sensory recovery by 36 hours and motor recovery by 3 years following a 3cm allograft [108] . Duel in 1934 reported 6 cases of 2-4cm thin nerve allografts to facial nerves, all producing excellent outcomes [109] . However the majority of investigators noted failures. Sander's review of 1942 showed in the 42 clinical cases undertaken by that time, only 8 had any improvement [110] . By 1950 most commentators were aware of the poor results and the pathological features of necrosis and cicatrization within nerve allografts such that clinical nerve allografting was condemned [111,112] .

Animal experimentation with peripheral nerve allografts (PNAG) continued in parallel with the clinical cases. Forsmann claimed PNAGs were more sucessful than xenografts in 1898 [113] . In the hundred years following this, the vast majority of investigators studying fresh PNAGs reported results of failure or rejection [97] . Some successes were claimed. Huber in 1919 and 1920 reported qualitatively excellent results based on histological features at 83 days with rabbit allografts [114,115] . Bentley in 1936 and 1940 with cat and primate allografts noted distal regeneration with histological and electrophysiological assessments at up to 500 days [116,117] .

Interpretation of results from allograft studies can be difficult as many experiments predate an understanding of immunology.

In 1915, Ingebrigsten observed an allograft lymphocyte infiltrate around 11-12 days and was the first investigator to attribute his findings to the host's immune response [118] . This reaction was more marked than that seen in autografts and caused Schwann cell destruction. Wallerian degeneration was similar in autografts and thin allografts, but not in thick allografts [119] . Sanders also associated larger PNAGs with greater rejection [120] , and Levinthal noted results equivalent to autografts with fascicular allografts but not with whole PNAGs [121] . Zalewski demonstrated regeneration across short but not long allografts with minor histocompatibility disparity, while no regeneration occurred with MHC disparity despite graft length [122-126] .

Timing also seemed to be an issue. Regenerating axons were identified within allografts and distal host nerves but their appearance was delayed compared to autografts [116,117,127-129] . In a rabbit study, Gutmann (1942) blamed an early host reaction to the allograft for the delay and subsequent poor function despite identification of axonal regeneration [130] . While in 1943 he observed delayed regeneration in the same model but results otherwise were similar to autografts [43] .

Davis also noted conflicting results from two similar experiments. In 1945 he found consistently inferior results with allografts when compared with autografts and simple coaptations in a feline model, including greater fibrosis, muscle atrophy and trophic ulcers [131] . His 1950 study however describes excellent results although central allograft necrosis was identified [112] . The main difference between the studies was that the former lasted up to four months and the latter up to one year. Of all the studies described, despite their flaws, generally the ones showing the most promising allograft results were undertaken over longer time periods than the majority of studies showing rejection e.g. Bentley 1940 – 200 days [117] ; Gutmann 1942 – 500 days [128] ; Bain and Fish 1992 – 12 months. Bain and Fish conducted primate studies and noted rejection at 6 months with equivalent results to autografts by 12 months [132,132,133] .

Like Gutmann, Midha demonstrated equivalent numbers of axons in PNAGs and autografts but poorer functional outcome [134] . An immunological explanation was the most likely cause of this and the overwhelming number of failed allografts. This was confirmed by evidence showing superior regeneration from related donors and recipients. Hirasawa observed regeneration was superior in littermates and parents of his rabbit model than in unrelated animals [135] . Singh matched outbred dogs and reported the degree of MHC difference led to PNAG rejection rather than minor histocompatibilty disparity [136,137] , while Schaller also noted that rejection and quality of regeneration varied with the degree of histocompatibility disparity in an inbred rat model [138] .

1.6.2.1 Immunology and graft rejection

Rejection was not understood as a concept until the immune system had been identified as the source of the process that destroyed "foreign" tissues. The use of donor tissues was viewed as a potential therapy for many life threatening conditions. Technically, transplantation has been achievable for many structures for decades. However the usual response of recipients was that they mounted an

inflammatory-type reaction which destroyed the transplanted tissue. The host organism rejected it.

While investigating the use of allogenic skin in burned patients in Glasgow during World War II, Tom Gibson, a pioneer in Plastic Surgery, observed accelerated reactions following the second application of allogenic skin from the same donor. Working with Peter Medawar, a pioneer of immunology, Gibson and Medawar confirmed rejection occurred more rapidly and aggressively during this "second-set" reaction. This observation is generally seen as being the cornerstone of the development of immunology. Medawar subsequently identified that foreign tissue was destroyed by the host following an active systemic immune reaction, exhibiting both specificity and memory against the donor tissue [139] . Following on from this, relatedness between host and donor was seen to be paramount to graft survival and culminated in the present concept that rejection is due to disparity of major histocompatibility (MHC) loci between the donor and recipient tissue, plus any tissue-specific or minor histocompatibility loci [140] .

Allorecognition is the first stage in rejection, which can be direct or indirect. In the former, host T lymphocytes (CD4+) are capable of directly recognising allogeneic class II MHC molecules. For indirect allorecognition the host antigen presenting cells have to process and present donor MHC molecules [141,142] . Further signals from host and donor antigen presenting cells may be required for T cell activation. T –cell mediated immune reactions (cellular immunity) are believed to feature most prominently in rejection but antibody-mediated reactions (humoral immunity) are also implicated [140] . These two systems form the effector host response. The T cell sub-sets CD4+ (T helper cells) and CD8+ (cytotoxic T cells) interact with antigen associated with MHC class II and I respectively. Class II mechanisms are more readily implicated in rejection.

Once activated, CD4+ cells secrete cytokines which initiate cascade reactions signalling graft infiltration by other immunocompetent cells e.g. macrophages [143] Cytokines are also involved in upregulation of MHC expression amplifying the immune reaction further [144,145] . Acute rejection is mainly mediated by cellular immunity with the graft infiltrated by CD4+ and CD8+ cells, along with macrophages, and subjected to direct cytotoxic cell insults and from secondary mediators of inflammation [145,146] . Antibodies may also be involved against blood group and MHC antigens. These cause complement-mediated lysis or damage via activation of cytotoxic macrophages and lymphocytes [140,147] . Antibody reactions are most problematic when the graft is directly connected to the host vasculature. Cellular immunity directed against graft endothelial cells [148] leads to microvascular occlusion with ischaemia and necrosis [146] the final common path of allograft destruction.

Rejection is recognised in three forms: hyperacute occurs in minutes to hours and is mediated by host preformed antibodies; acute rejection occurs 5-21 days post grafting and is due to donor sensitization, previous sensitization produces accelerated rejection; and chronic rejection develops late and is caused by a disturbance in graft/host relationship breaking down T cell tolerance to the graft [140,143] .

1.6.2.2 Nerve immunogenicity

Some of the findings suggesting an immune response in nerve allografts have already been described. MacKinnon in 1982 in a ^{51}Cr release assay used donor specific splenocytes to challenge graft recipients at various times post transplant to study the host reaction. Host recognition of nerve allograft was at day 8 in hosts with a MHC disparity, while delayed to day 80 where a minor difference existed[149] .

The inflammatory infiltrate associated with PNAG was noted to increase while axons and Schwann cells decreased [150,151] . The inflammatory infiltrate included lymphocytes, macrophages and plasma cells [152] , and was initially epineurial before becoming endoneurial and associated with the microvasculature [150,151] . The nerve architecture was disrupted with rupture of Schwann cell basal lamina tubes, as identified by electron microscopy [152] , which hindered axonal regeneration. Allograft vascularity was also affected. Described as good at day 7, it steadily reduced from day 14-30. Peak cellular infiltrate assessed from aspiration of fluid around the graft occurred at day 9. The proportion of CD4+, and CD8+ lymphocytes plus cells expressing MHC class II was greater than in corresponding isografts [153] . Hare also confirmed a marked infiltrate of similar lymphocytes in allografts in an outbred ovine model, along with increased MHC class I and II expression on infiltrating and graft cells compared to autografts [154] .

The source of PNAG antigenicity has been investigated partly to try to find a method of blocking its effects. As previously described, a nerve is made up of several components. The structure is of connective tissue origin containing collagen, elastin, reticulin, tubulin and laminin. Within this are axons, non-neuronal cells, largely Schwann cells, but also immunocompetent cells such as macrophages, blood vessels and lymphatics. The lack of a lymphatic system can confer a degree of immunological protection as in the cornea and some CNS sites [140] , but only the endoneurium has no lymphatics in nerve.

In nerve allografts increased expression of MHC II has been noted on endothelial and perivascular cells within the time period of acute rejection [151,155] and in cultured endothelial cells [148]. During studies on nerve grafts, Best identified that revascularisation by longitudinal inosculation began around 24 hours and was present by 48 and 72 hours within the epineurium and endoneurium respectively [156] . Therefore donor endothelium is exposed within 72 hours to the recipient's circulation and immune system. Endothelium is known to be highly immunogenic in

solid organ transplantation work [146] and therefore is highly likely to be involved in nerve allograft rejection.

Perineurial cell and fibroblasts of the epineurium were also investigated as antigenic components of nerve as individual fascicles had been found to elicit less reaction that whole nerve grafts [121,157] . Acellular freeze-thawed nerve allografts did not incite an obvious immune reaction [158,159] , although in experiments with previously sensitized hosts a reaction did occur indicating weak antigenicity within the nerve skeleton [160] .

The other most likely antigen presenting cell in nerve and a likely focus of immunoreactivity are the Schwann cells. Myelin itself was implicated but predegenerated allografts containing no myelin were still found to reject [161] .

Many authors observed Schwann cells in nerve allografts were destroyed. In early reports this was just presumed but since the technology has existed to identify Schwann cells by electron microscopy and immunocytochemistry this feature has been confirmed [102,151,152,161,162]. Using interferon gamma (IFNγ)[163-165], and tumour necrosis factor beta (TNFβ)[164] , investigators have found inducible but not constitutive expression of MHC class II expression on Schwann cells in vitro. Additional experiments using specific antibody against IFNγ and inhibitors of IFNγ (hydrocortisone, cyclosporin, prostaglandin E_2) have produced inhibition of Schwann cell MHC class II expression, further implicating IFNγ in class II upregulation [166].

Evidence has also been found of MHC class II on the surface of Schwann cells in vivo [167]. Lassner and Grochowicz identified MHC class II on Schwann cells in

rejecting allografts but not on autografts [151,168] . However others have identified this following simple injuries [169,170]. Class II expression has been identified on Schwann cell basement membrane and within endocytotic vesicles giving a strong indication that they behave as APCs (antigen presenting cells) [171,172] and they are known to be able to phagocytose myelin and axonal debris during Wallerian degeneration [173]. Bergsteinsdottir also reported that Schwann cells could produce interleukin-1 (IL-1) following stimulation, and IL-1 immunolabelling was predominantly intracellular [174]. Wekerle caused T-cell proliferation and clustering around MHC II expressing Schwann cells by stimulating them with IFNγ, whereafter they presented both exogenous and endogenous antigen to antigen-specific T-cell lines [163], given further evidence suggesting Schwann cells have a role in antigen presentation, making them a target for rejection.

An alternative path has also been suggested by experiments but this lies contrary to the classical view that MHC class I and class II present endogenous and exogenous antigens respectively. Schwann cells could also be direct targets for CD8+ cells. Schwann cells have been shown to express class I antigens constitutively and after IFNγ stimulation [166,171] , and Steinhoff identified that IFNγ class I induced Schwann cells underwent cytolysis by antigen-specific CD8+ cells following exposure to exogenous antigen [175] . This alternative pattern of MHC class I and II function has also been noted in other systems [176] .

It is likely from the large body of evidence that PNAG endothelium and Schwann cells are the major APCs and foci of the rejection response along with some minor antigenicity from structural elements.

1.6.2.3 Immunosuppression

There are two available routes to reducing the immune reaction to nerve allografts: specific or general methods. Ideally specific methods would make this conduit more agreeable as the host would not require to be subjected to agents which may have deleterious effects on areas not concerned with the allograft.

Specific methods have included graft pretreatments, alone or in combination and graft ensheathment. In the latter the aim was to physically protect the allograft from host infiltration. Millipore and frozen/irradiated PNAG [177] and aldehyde tanned collagen with lyophilised PNAG [178] were used, but both failed.

Pretreatments were aimed at reducing the antigenicity of the allograft. Many of these experiments predate immunological knowledge and suffered from lack of experimental rigour. Methods of pretreatment include chemical storage, in situ predegeneration, freezing, lyophilisation (freeze-drying), freeze thawing, and irradiation. Some successes were claimed with these measures alone, or in combination, but generally the majority failed in comparison to autografts, although the methods may have succeeded in improving the regeneration or reducing the reaction compared to fresh PNAG [97]. Storing in UWCSS (University of Wisconsin Cold Storage Solution) showed some promise following on from the findings of Sanders, with reduced Schwann cell viability with time leading to reduced host reaction and improved regeneration through the PNAG [179]. However, Evans showed that inbred rat PNAG, after 3-5 weeks cold storage in UWCSS, regenerated similarly to fresh PNAG but less than autografts [180], although similar storage in sheep had shown a reduced host lymphocyte reaction compared to fresh PNAG [154]. Most methods (freeze-thawing, lyophilisation) have rendered the PNAG acellular although preserving the endoneurial tube architecture to aid axonal regeneration by both mechanical and trophic support [159,181,181,182,182] . However without Schwann cells axonal regeneration has been shown to be reduced in autografts and allografts, and this influences the regeneration potential of any

conduit [75,76,158,183] . Lyophilisation was observed to reduce host response but although regeneration occurred it was inferior to autografts [178,184,185] . Similar results were found with freeze-thawing [158] although results in short grafts (< 4cm) were more promising [180,186] . With irradiation, high doses were required to reduce PNAG antigenicity which also led to reduced regeneration and increased fibrosis [187,188] . Irradiation was often used in combination with freezing. Some successes were noted [99,189] but were poorly designed and controlled. Other, well controlled studies confirmed that freeze/irradiated PNAG supported regeneration better than fresh PNAG but consistently observed inferior regeneration to autografts [184,190-192] .

Predegeneration was also often combined with other pretreatment methods. In situ nerve predegenerative has no real clinical relevance and had been shown not to enhance regeneration within allografts or autografts [154,161,191,193] . The clearance of myelin and proliferation of Schwann cells may have been thought to be beneficial before the antigenicity of Schwann cells was fully appreciated, but using it along with a method to render the graft acellular seems counterproductive [184,190] .

The major improvement in the prospects of all transplantation came with the advent of immunosuppressant agents. These would produce non-specific, generalised host immunosuppression. After their introduction it became apparent very quickly that these treatments offered no miracles. The immunosuppression they induced meant that although the graft was protected from the host, the host was exposed to other assaults such as infections and malignancy. Added to this were the unwanted drug side effects from the uncomfortable nuisance effects of nausea, weight gain, and itch through to the serious side effects such as liver and renal toxicity. Hippocrates is credited with saying that "for extreme illness, extreme treatments are fitting" [104] . While this stance may be appropriate regarding life threatening conditions, it may not be justifiable to risk someone's life and health to improve disability and morbidity. This is the case with nerve allografts. Many

people, medical and lay, find it unethical to transplant allogenic nerves if long-term immunosuppression is required.

Donor specific methods of reducing rejection result in tolerance of the host towards the donor antigens but retained ability to respond to all other antigenic challenges. Zalewski induced tolerance in neonatal rats by injecting them with the relevant F1 progeny lymphoid cells. This method has no clinical relevance, but regeneration and muscle reinnervation (in cases with distal coaptations) occurred. The tolerance was reversed using donor sensitized cells and rejection ensued, but axons were preserved and function recovered when host and donor had minor histocompatibility differences [194] . However, without a distal repair or with major histocompatibility differences all was lost [125,195] . These findings highlight an important feature of nerve allografts which is different from other transplants: although the conduit is allogeneic, the axons are host derived therefore rejection long-term of the PNAG may not be so important if the axons are not permanently harmed by rejection.

Non-specific methods of reducing the host's immune response also reduce the ability to respond to all antigens not just those in the transplant. These agents are usually required long term and their development and use has followed solid organ transplantation. The initial drugs used in PNAG experiments were the antimetabolites 6-mercaptopurine, actinomycine-C, and methotrexate; along with prednisolone, hydrocortisone, and azathioprine [99,185,189,196] . It was not until the discovery and clinical use of cyclosporin A (CyA) that organ transplanting moved from the laboratory to clinical reality, with a risk/benefit ratio more acceptable for the patient [104,197-199] .

1.6.2.4 Cyclosporin A

Cyclosporin A was isolated from a Norwegian soil sample in 1970 by scientists working for the drug company *Sandoz*. It is derived from a fungus *Tolypocladium inflatum Gams* and its immunosuppressant activity was identified and developed by the immunologist Jean Borel [200]. The benefit of CyA over other immunosuppressants was its selective action on T-cells [198,200] . CyA blocks IL-2 production from CD4+ (Th) cells, inhibits T-cell proliferation, binds immunophilin and inhibits calcineurin action [201]. Its profound inhibition of CD4+ and CD8+ activity and promotion of suppressor T cell function make it a powerful single agent immunosuppressant [197,202]. That CyA could immunosuppress adequately as a single agent was of interest to workers in nerve allografting. Also, it became evident that if immunosuppression could succeed in protecting axonal regeneration through the allograft at least similar to that encountered through autografts, then as the axons were host derived, perhaps CyA immunosuppression would be required only short-term. Following axonal regeneration the allograft conduit theoretically could be rejected as long as regeneration sustained no irreversible deleterious effects.

Studies with long term CyA immunosuppression identified successful regeneration in comparison to non-immunosuppressed PNAGs [123,124,153,192,203-205] and equivalent regeneration to autografts [123,134,156,192,196,206-210] using histological qualitative and quantitative assessments [129,134,207,208] as well as tests of function [123,129,132,211]. Ishida also used immunological assessments to demonstrate suppression of both cellular and humoral immunity towards a donor-specific challenge in CyA treated mice with PNAGs [208] . Only one study identified poorer regeneration with CyA. Ansselin used "Trembler" mice and found they rejected allografts on doses of 10-15mg/kg/day of CyA [212], however assessments of electrophysiology and morphometry were compared with normal nerve, not isografts or autografts, thereby enhancing any deficiencies in regeneration. The CyA had been administered orally. This route is associated with extremely variable bioavailability and low peak levels both in animals [213] and humans [214] . CyA is non-water soluble

and the compound has to be emulsified in oil for administration. This is notoriously difficult and may add to the problems of ensuring adequate doses in small animals (see Chapter 2).

Investigators were aware of difficulties with CyA, and needed an easy route of administration for the animal models which would also ensure the desired immunosuppression. Bain identified the minimum effective dose required to prevent histologically evident rejection and immunologic reaction of sensitized hosts to a donor-specific antigen challenge by mixed lymphocytic culture. This is 5mg/kg/day of CyA [206] .

The use of temporary immunosuppression has also been investigated to test whether regeneration can be sustained, while withdrawing immunosuppression would reduce the long-term risks of it to the patient. The experimental findings have drawn many conflicting conclusions, some of which can be traced to the experimental designs. Complete rejection following immunosuppressant withdrawal has been observed [123,125,168,195,204], as has no evidence of rejection [185,196,215] . Other groups identified intermediate reactions with incomplete loss of function and subsequent recovery, along with weaker and delayed histological changes of rejection [134,192,209-211,216-223].

Factors contributing to this mixed picture include: short follow-up, no distal neurorrhaphy, small sample size, inadequate immunosuppression, lack of adequate controls and quantitative outcome parameters. From experiments where no rejection was reported post-immunosuppression, MacKinnon did have results inferior to autografts at the 80-day study end-point (using azathioprine /hydrocortisone) and could have missed rejection within this time period [196] , while Pollard employed inadequate follow-up [185,215]. Of the experiments where rejection was shown Zalewski performed only a proximal neurorrhaphy thereby denying the

regeneration potential of his model the proven neurotrophic benefits of the distal host nerve [123,125,195,204] . Also without host axons traversing the graft to reach target organs the whole regenerated system would be theoretically more susceptible to irreversible assault.

More recent studies have indicated that a rejection reaction does occur following cessation of, in these cases CyA, but that the effects appear to be recoverable [134,209-211,216,219-221]. In MacKinnon and Ishida's studies analyses were by histology and functional assessments and immunosuppression was for 12 and 8 weeks respectively, with correspondent final assessments at 36 and 20 weeks [209,216]. In MacKinnon's study histology revealed evidence of remyelination at 20 weeks therefore it was hypothesized that the remyelination occurred secondary to migration of host Schwann cells into the nerve allograft following rejection of donor Schwann cells [216]. These two studies however suffer from the lack of autograft, continuously immunosuppressed allograft and non-immunosuppressed allograft controls. Midha redressed these problems in his study where inbred rats were immunosuppressed for 12 weeks to allow innervation of target organs, which was confirmed with functional analyses. Following CyA withdrawal demyelination occurred with moderate axonal loss and a decrease in walking track and electrophysiological parameters. Of the significant proportion of axons which prevailed, remyelination occurred plus regeneration of the damaged axons. Finally the functional and histologic parameter were similar to autograft and continuously immunosuppressed allografts and superior to non-immunosuppressed allografts controls [134,218] .

Seven reported clinical cases were undertaken between 1988-1998. CyA was the main immunosuppressant used although it was replaced by FK506 (tacrolimus) for the most recent two cases. All patients were young with large defects in major limb nerves, who had sustained their injuries on average four months before reconstruction. Cabled allografts were utilised and immunosuppression was

maintained for 12-26 months. All but one case has shown signs of nerve regeneration, with sensory recovery generally exceeding that of motor function. Protective sensation has been universally achieved and the first two patients are described as being able to walk unaided and play sports. The single failed case was attributed to subtherapeutic CyA and rejection [217,224,225] .

Newer immunosuppressants are increasingly available with stronger immunosuppressant activity and less toxicity and side-effects. Tacrolimus (FK506) has been used in allograft work and has produced results similar to those seen with CyA. So far, none have induced tolerance despite the promise shown in some studies [226-230] . For nerve allografting the main dilemma remains not the technical ability, or even the ability to achieve immunosuppression, it is the ethical matter of whether potentially life threatening treatments should be used in the treatment of non-life threatening conditions.

The recent clinical cases of composite transplantation of hands has brought these issues to the fore again [231-237] . If the ethics are not clear cut for these debilitating and cosmetically obvious disabilities, then they are even more unclear for isolated nerve injuries. However the fate of nerve allografting and composite grafting, especially of the upper limb are inextricably linked: without improvement in nerve allograft regeneration a major aim of the hand transplant will have failed even if it technically survives. This is because the role of the reconstructive surgeon is to restore appearance and function; if the hand is without function it is virtually useless and also has an obviously unusual appearance. A modern prosthesis, and the potential offered in this respect from microchip technology and bioengineering maybe more likely to provide service to the effected individual.

1.7 HYPOTHESIS AND AIMS

At present conduits of nerve origin provide the best substrate for axonal regeneration. However, in nerve allograft, Schwann cells are antigenic and are rejected leaving weakly antigenic endoneurial tubes, which may support host axon regeneration. It is still unclear whether this antigenic reaction has to be permanently controlled with immunosuppression to obtain best regeneration results. However, for nerve allografts to be ethically more acceptable, the requirements for immunosuppression might need to be reduced while maintaining regeneration at least similar to autografts.

Regeneration can be improved within acellular organic and synthetic conduits by supplementing the availability of Schwann cells [61,62,75,238-240] or the neurotrophic factors they produce [4,52,65,241-243] . A simple method of achieving this was demonstrated by Maeda and Whitworth [244,245] . They noted improved axonal regeneration through silicone tube and muscle grafts following interposition of autologous nerve grafts between sections of these two conduit materials (called *stepping-stone* or *sandwich* grafting).

It is hypothesised that similarly, a host sandwich allograft (formed from a chimeric structure of host and donor nerve segments sutured orthotopically together) will provide a depot of non-allogenic Schwann cells within a nerve allograft to provide neurotrophic support to regenerating host axons to compensate for the rejection of allogenic Schwann cells (see Chapter 2). In addition, the increased proportion of host Schwann cells might reduce the antigenicity of the whole graft structure. Thus any post-rejection axonopathy and delayed regeneration resulting from rejection might also be reduced, leading ultimately to reduced requirements for immunosuppression.

The aims of this thesis were:

(1) To study and compare in an animal model the early time course of regeneration within immunosuppressed and non-immunosuppressed nerve allografts in comparison to nerve autografts, with particular reference to the progression of axonal regeneration, inflammation, vascularisation and Schwann cell response.

(2) To investigate the possibility of augmenting regeneration within a non-immunosuppressed nerve allograft by utilising a sandwich grafting technique, with emphasis on the effects that the autologous sandwich graft segment has on Schwann cells and inflammation within the early post-engraftment period.

(3) To assess the requirements for long-term immunosuppression by comparing simple and sandwich nerve allografts, along with equivalent non-allogenic controls. Analysis of axonal regeneration in relation to end organ reinnervation would be used as indication of functional recovery.

CHAPTER TWO

Materials and Methods

2.1 EXPERIMENTAL PROCEDURES

2.1.1 Animal model and anaesthetic technique

All nerve graft experiments conducted in this project involved eight week old female inbred Lewis (Lew) and Dark Agouti (DA) rats. These strains are known to be incompatible at both major and minor histocompatibility complexes [246-248] . This rat combination has been used by previous authors in the fields of peripheral nerve allografting [220,247] . Also, the use of the rat allows a better comparison with the work of other authors, especially when investigating a new technique [249] .

Animals were anaesthetised using inhalational induction with Enflurane [Abbott Laboratories Ltd, UK], followed by an intramuscular injection of *Hypnorm* 0.3mg/kg [fentanyl citrate: 0.315mg/ml and fluanisone: 10mg/ml, Janssen Pharmaceuticals Ltd.], and 2.5mg/kg of intraperitoneal diazepam [Phoenix Pharmaceuticals Ltd.]. All procedures were carried out in compliance with personal and project licenses issued under the Animals (Scientific Procedures) Act 1986.

2.1.2 Operative procedure

General principles of surgery were employed to avoid compromising nerve vascularity and to ensure atraumatic tissue handling. All microsurgical procedures were undertaken using an operating microscope [Wild Heerbrug Ltd., Germany], which also provided attachments for per-operative photography.

Experiments were carried out on the animals' right sciatic nerves (Figure 2.1). The nerve was exposed through a lateral thigh incision utilising an intermuscular approach between the superficial gluteal and the biceps femoris muscles. A 10mm segment of sciatic nerve was measured by a template and removed to produce a standard gap size. The proximal edge of this segment lay just distal to the consistent vascular pedicle which the sciatic nerve receives in the upper thigh. Before grafting, the proximal and distal nerve stumps were mobilised for no more than 5mm to preserve vascularity.

The nerve gap was repaired using one of the five graft techniques shown in Table 2.1 and Figure 2.2. These grafts form the basis of the study groups used throughout this project. The graft sizes are detailed in the relevant chapters.

Table 2.1 Experimental graft types under investigation

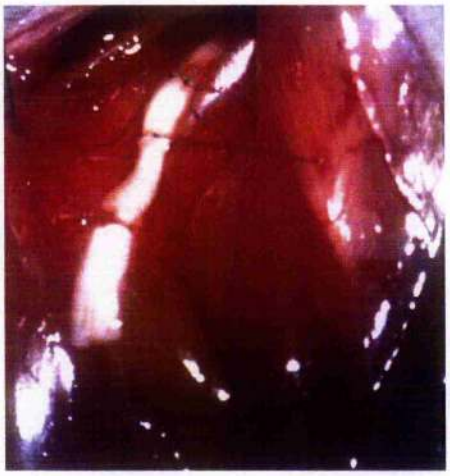
Graft	Host	Donor
Nerve autograft	Lewis	autologous Lewis
Nerve isograft	Lewis	isogeneic Lewis (Lew)
Nerve allograft	Lewis	allogeneic Dark Agouti (DA)
Nerve sandwich allograft	Lewis	Dark Agouti, with autologous Lewis central segment
Nerve sandwich isograft	Lewis	isogeneic Lewis,with autologous Lewis central segment

The nerve graft was obtained from a branchless portion of donor sciatic nerve. Epineural repairs were carried out at all neurorrhaphies using interrupted sutures of 9/0 monofilament nylon [Ethicon Ltd., UK]. Care was taken to avoid nerve rotation, misalignment and axoplasm herniation. In the case of the sandwich grafts, each 5mm segment was sutured in continuity from proximal to distal. Nerve regeneration in the rat is widely recognised as being more efficient than other higher species such that critics [52,211,250] have maintained that a 10mm gap may be bridged without repair in this model, therefore an un-repaired 10mm gap was also undertaken as a control.

Figure 2.1 Operative Series of Sandwich Graft Construction

a) Proximal nerve stump

b) Nerve graft coapted. Will be halved before addition of host segment



c) Middle host segment in place with adjacent final segment

d) Whole construct in continuity

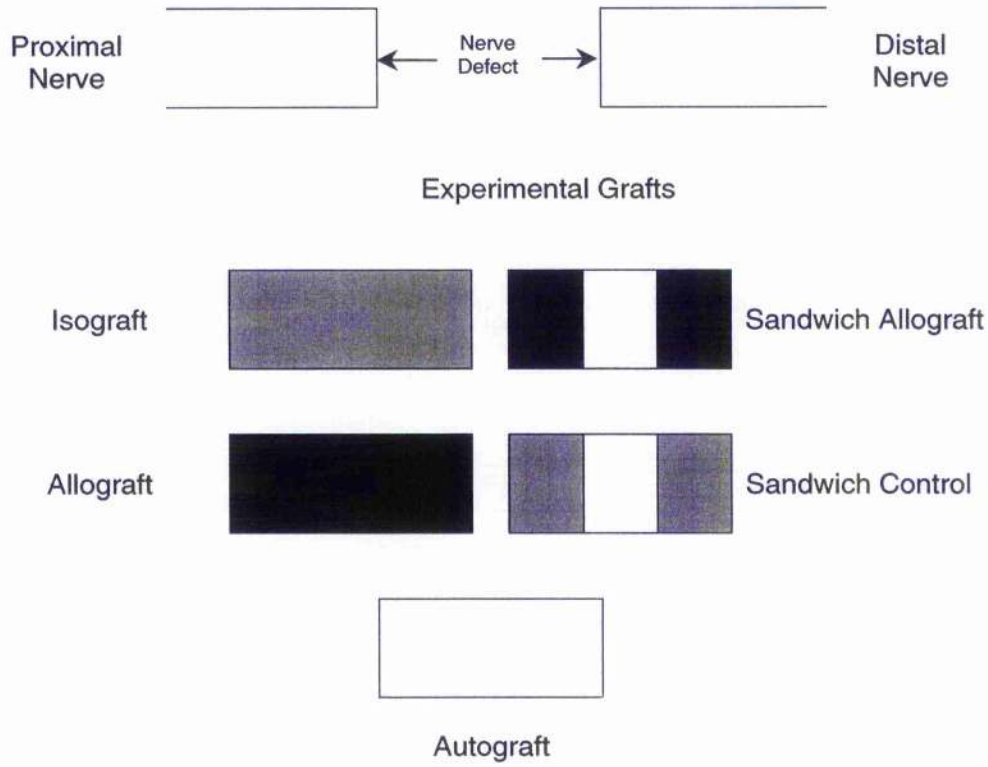
Orientation is with proximal uppermost and distal at the base of the frames.

The wounds were closed using horizontal mattress sutures of 3/0 silk [Ethicon Ltd., UK]. Animals receiving cyclosporin (CyA) were injected subcutaneously at the end of the procedure (see Section 2.2). All animals received 5ml of 0.9% saline [Baxter

Ltd., UK] subcutaneously to prevent the likelihood of dehydration. The rats initially recovered in single cages in a warm room. They were returned to their six animal group cages within the holding facility 24 hours later. The rats were housed under standard conditions with free access to food and water for the duration of the experiments. Every effort was taken to minimalise animal distress and numbers.

Each experimental group was comprised of six animals which was the minimum number of animals required to allow valid statistical analysis. This number was established during planning of the experiments following discussion with a statistician (see section 2.4) and based on findings from previous work [64,245,251,252]. The experiments involved comparing the graft types mentioned previously (Table 2.1) over time points ranging from 7 to 21 days in the short term, to 32 weeks for long term experiments.

Figure 2.2 Diagram of Experimental Groups



Technical errors and variation during the surgical procedures were reduced to a minimum as only one operator was involved, carrying out a total of more than one thousand microsurgical procedures. The operator had previous microsurgical experience ensuring that the procedures were performed in a reproducible and optimal manner. All the rats used were from inbred colonies, of single sex and similar age at the commencement of the experiments, therefore minimising any variation in nerve regeneration attributable to operator or model variability. All animals were acclimatised for two weeks prior to surgery, in accordance to Home Office guidelines, thus minimising any variations attributable to animal stress.

2.1.3 Tissue harvesting and preparation

Animals were sacrificed at the end of each experiment using high dose Enflurane inhalation. The right sciatic nerves containing the graft segments were removed *en bloc*. These specimens were trimmed using a plastic template so that 5mm of host nerve was included proximal and distal to the grafts. The specimens were pinned to card for orientation, and to avoid shrinkage during fixation.

Preparation for immunohistochemical staining involved fixation in Zamboni's solution (2% paraformaldehyde and picric acid in PBS, pH 7.4 - Appendix 1) for 6 hours at room temperature (for fresh tissue less than 5mm thick: ratio of fixative to tissue at least 10:1). Afterwards, the specimens were rinsed in phosphate buffered saline (PBS) containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide (PBS-sucrose - Appendix 1) at least three times over the following 3 days while the specimens were stored at 4°C. Rinsing was complete when the rinsing solution lost the yellow tinge from the Zamboni's fixative and the tissue had sunk within its container. Thereafter, any sutures were removed under magnification prior to preparation of cryostat blocks, where liver segments were used to identify the proximal ends of the sciatic nerve/graft complexes. The tissues were blocked in OCT [Optimum Cutting Temperature compound, Tissue-Tek, Miles Inc., USA] and were stored at -40°C while awaiting sectioning and staining.

A section of posterior tibial nerve was collected for myelinated axon counts from animals belonging to long-term 32 week groups. Preparation of these specimens was undertaken with the assistance of Dr. Padmini Sarathchandra, Department of Pathology, Northwick Park Hospital NHS Trust, Harrow. A 3mm section of this nerve was removed 5mm distal to the distal anastomosis and immediately fixed in electron microscopy grade 2.5% gluteraldehyde [Agar Scientific Ltd., UK] in 0.1M phosphate buffer (Appendix 1) overnight at 4°C before rinsing twice in 0.1M phosphate buffer. The specimens were fixed for 1 hour in 1% osmium tetroxide

[Agar Scientific Ltd., UK] in 0.1M phosphate buffer followed by washing in phosphate buffer (2x5minutes). Following dehydration in graded ascending acetones [Agar Scientific Ltd., UK], the specimens were infiltrated with an acetone/Araldyte mixture (ratio 1:1) [Agar Scientific Ltd., UK] overnight. Thereafter the specimens underwent two changes of pure resin before final Araldyte block polymerisation at 60°C for 18 hours. Transverse semithin (1μm) sections of the nerve were cut from the blocks and collected on glass slides. These sections were stained with thionin and acridine orange for analysis of myelinated fibres (Section 2.3.4). Similar portions of contralateral unoperated posterior tibial nerves, as measured from the sciatic notch, were harvested and processed as above to be used as controls.

Gastrocnemius muscle mass was measured as an assessment of end organ reinnervation in the long term experimental groups [64,65] . The muscles from both legs were approached via an extension of the original incision. The entire muscles were dissected cleanly and carefully under magnification and detached flush with bone at their origins and insertions. Each muscle was weighed to allow calculation of the percentage reduction in muscle mass between the operated and non-operated legs during the experimental period.

2.2 IMMUNOSUPPRESSION

The sole immunosuppressant used was cyclosporin [Sandoz Pharmaceuticals Ltd., UK](also known as cyclosporine and cyclosporin A). Two preparations were used: the parenteral formulation available commercially (pCyA) (see Section 2.2.3), and cyclosporin (CyA) base powder donated by Sandoz, along with instructions for its preparation and administration (Section 2.2.1-2.2.2). In the rat, previous work with cyclosporin [213] has led to the wide acceptance of a dose of 5mg/kg/day by subcutaneous injecton as suitable for immunosuppression in this model [209] [206,207,216] .The two preparations were used initially for financial reasons and

secondly because during the course of the experiments there was concern regarding the bioavailability of CyA solution prepared from the base powder (oCyA). This became evident after some of the rats began to develop encysted collections of oily fluid following administration of the powder preparation in the subcutaneous plane around the injection sites. Further details of the different effects experienced with the two formulations are given in Chapter 6.

2.2.1 Cyclosporin administration

Administration of cyclosporin was by daily subcutaneous injection and commenced the day preceding surgery. The injection sites were varied to avoid morbidity and maintain bioavailability. The dose each animal received was altered according to weekly weights [253] . A depot effect can occur when using this method of administration especially over prolonged periods [254,206] . This was also borne out by these experiments (see above and Chapter 6). Accordingly, CyA was administered on alternate days after the first twelve weeks of any long term experiments. Full precautions, in keeping with Health and Safety requirements were employed when preparing and handling these solutions.

2.2.2 Parenteral cyclosporin (pCyA)[Sandimmun]

Commercially produced vials of pCyA at 50mg/ml (in solution with 650mg polyethoxylated castor oil and 33% ethanol by volume) were diluted using 0.9% saline [Baxter, UK] to produce a solution of 10mg/ml as per the manufacturer's instructions (Sandoz Pharmaceuticals Ltd., UK; Ishida et al., 1993).

2.2.3 Oily cyclosporin preparation (oCyA)

Pure cyclosporin is a non-water soluble fine white powder. To achieve the best solution for *in vivo* administration, Sandoz instructed the preparation of a solution as follows: 100mg CyA dissolved in 165mg/ml of ethanol absolut, followed by 350mg/ml of corn oil and 385mg/ml of Labrafil M2125 CS [Gattefosse, France].

This is a derivatized vegetable oil comprising unsaturated polyglycolysed glycerides, donated by Alfa Chemicals Ltd., UK. These quantities (900mg) correspond to 1ml of primary solution. To this a further 9ml of corn oil was added to produce a final solution of CyA with a concentration of 10mg/ml.

2.2.4 Animal care during cyclosporin administration

Cyclosporin is a toxic agent associated with many side effects [253,255,256] , mainly related to hepato- and nephrotoxicity. One sensitive indicator of CyA toxicity in rats relates to weight, either failure to gain weight or weight loss [253,254] . Animals were weighed weekly and were observed for signs of ill health on a daily basis. Generally, the animals were observed for changes to their limbs, skin and behaviour (see also Chapter 6). Dehydration can precipitate CyA toxicity, therefore efforts were made to minimise the likelihood of this occurring, especially peroperatively.

2.2.5 Cyclosporin monitoring

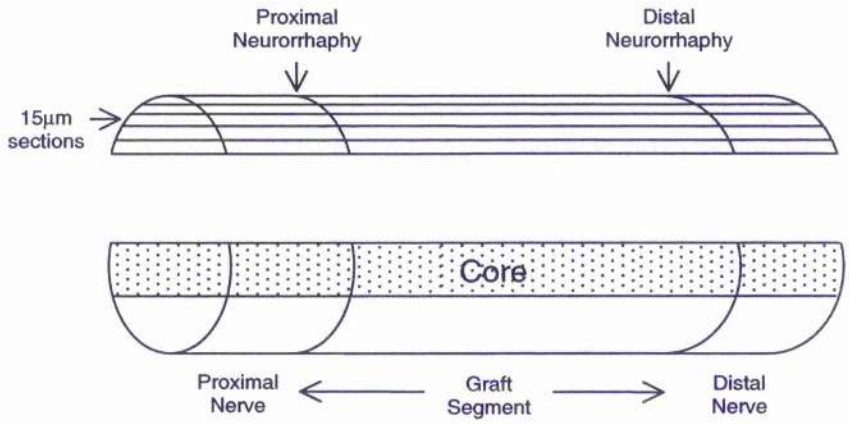
Circulating cyclosporin levels were measured from whole blood samples taken by venepuncture of the tail veins or by intra-cardiac puncture following terminal anaesthesia. Samples were collected in paediatric EDTA and lithium heparin coated tubes. Analysis for creatinine, alkaline phosphatase, alanine transaminase (ALT) and aspartate aminotransferase (AST) were undertaken as tests of hepatic and renal function [253] . [257] Cyclosporin was measured from whole blood using fluorescence polarization immunoassay (FPIA) technology [Tdx Cyclosporine Monoclonal Whole Blood Assay, Abbot Laboratories, USA]. This system is capable of detecting concentrations with 95% confidence for samples containing greater than, or equal to 25ng/ml of cyclosporin. Mr. Mark Dunsford and colleagues, Department of Chemistry, Guy's Hospital, London carried out these biochemical analyses.

2.3 MORPHOLOGICAL ASSESSMENT

2.3.1 Cutting techniques

Specimens of the host nerve/graft complex were cut in 15μm longitudinal sections. To ensure that a similar representative portion of the nerve/graft complex was examined in all samples, initially one specimen was sectioned completely and stained. From this it was determined that at least 15 sections needed to be cut before the core within the nerve/graft complex was reached (Figure 2.3). Once reached, the core sections to be stained were collected sequentially on alternate Vectabond [Vector, UK] coated slides (Appendix 2), each slide was subsequently processed for immunostaining. For each antiserum, or histological stain, two slides with three sections each were utilised. Sections were allowed to dry for 4-6 hours at room temperature to reduce section loss during the immunostaining.

Figure 2.3 Diagram explaining the graft complex cutting technique



2.3.2 Immunohistochemical staining

The various staining techniques employed were carried out using a routine protocol. Analysis of coded samples by Dr. Giorgio Terenghi, Reader in Surgical Sciences (Blond McIndoe Centre, University College London) confirmed the consistency of staining. All analyses were conducted on coded slides which gave no indication of the tissue of origin. The image analysis of immunohistochemical staining is an established method of quantitative analysis. All analyses were carried out by the author and results were found to be consistent with co-workers using similar tissues and techniques [60,63-65] . A known positive control was included in each staining batch to confirm the reliability of the technique [258] .

2.3.2.1 Indirect immunofluorescence method

Indirect immunofluorescence was used to stain the longitudinal nerve/graft sections as this technique was found to give optimal contrast and allowed the best definition of regenerating axons within the grafts.

After drying, the area around the sections was marked using a hydrophobic pen [DAKO Ltd., UK] to contain the antiserum when applied. The sections were then immersed in PBS containing 0.2% Triton-X [BDH,UK] (Appendix 1) for one hour at room temperature to achieve tissue permeability. This was followed by rinsing in PBS (3x3 minutes) before application of the primary antibody (Section 2.3.3) within a humid chamber. Incubation was overnight (16-20 hours) at 4°C. The low temperature incubation used here maximises specific binding while minimising non-specific background staining.

On sections where fluoroscein isothiocyanate (FITC) conjugated antibodies were used as the second layer (see Section 2.3.3), additional counterstaining with pontamine sky blue was carried out for thirty minutes at room temperature following Triton-X permeabilisation, in order to reduce background fluorescence.

Additional PBS rinses (3x3 minutes) were also carried out before application of the primary antibody.

On day two, sections were washed in PBS (3x5 minutes) prior to incubation with the secondary antibody (1 hour at room temperature). During this time the humid chambers were protected from external light to preserve the fluorescent markers. Final rinses in PBS followed (3x5 minutes) with the addition of a penultimate rinse in Tween (1/4000) in PBS for one minute to remove any excess unbound secondary antibody. Slides were mounted using PBS/glycerol (Appendix 1) containing 2.55 (w/v) 1,4-diazabicyclo (2.2.2) octane [Aldrich Chemicals, UK] as an anti-fading agent. Slides were stored in darkness at 4°C ready for viewing.

2.3.3 Antibodies

Antibodies to several peptides and proteins were used to detect regenerating axons, Schwann cells, macrophages and blood vessels within the host nerve/graft complex.

The antisera used in this study and their target antigens are specified below:

1. Panaxonal marker of neurofilaments (PamNF); a cocktail of antibodies to heavy molecular weight phosphorylated neurofilament proteins which localise large and small calibre axons. PamNF acts as a general axonal marker which defines functioning axonal material and was used to assess axonal regeneration in the various nerve grafts employed in this project [259].

2. S-100; a specific Schwann cell cytoplasmic antigen [260]. This antisera identifies the presence and progression of Schwann cells within the grafts, although it does not distinguish their origin.

3. ED-1; a specific marker for rat macrophages. This antiserum was used to assess macrophage invasion into each graft as an indication of the degree of inflammation elicited [63,261].

4. von Willebrand factor (vWF); a specific endothelial cell surface antigen. This antiserum was used to identify blood vessels and the pattern of graft vascularisation [60].

The specific dilutions of the antisera and their suppliers are detailed in Table 2.2. All primary antibodies were diluted with a PBS solution (Appendix 1) containing 0.03% Triton-X [BDH, UK], 0.1% bovine serum albumin [Sigma Chemicals Ltd., UK], and 0.1% sodium azide [BDH, UK]. Second layer antibodies were all diluted with PBS only.

Table 2.2 Antisera specifications

Primary antibody	**Dilution**	**Identifies**	**Source**
PamNF (mouse monoclonal)	1/2000	axons	Affiniti, UK
S-100 (rabbit polyclonal)	1/1200	Schwann cells	DAKO, Denmark
ED-1 (mouse monoclonal)	1/800	macrophages	Serotec Ltd., UK
vWF (rabbit polyclonal)	1/1200	endothelial cells	DAKO, Denmark
Secondary antibody	**Dilution**	**Conjugate**	**Source**
Goat anti-mouse	1/100	Cyanine-3 (red)	Amersham, UK
Goat anti- rabbit	1/100	FITC (green)	T.C.S. Biologicals, UK

2.3.4 Thionin-Acridine Orange staining

Semithin (1μm) sections from the posterior tibial nerve distal to the graft segments were treated with thionin blue and counterstained with acridine orange (Appendix 3) to stain myelin sheaths around the regenerated nerve fibres. Myelin stains dark blue. From these, number of myelinated fibres, their size and myelin thickness could be measured (Section 2.3.5). Three slides, with a minimum of six sections on each, were prepared and stained from each animal.

Following fixation as already described, sections were collected on plain glass slides to be stained. The underside of the slides was flamed briefly ten to twelve times to provide section adherence before staining. Fresh thionin was applied to the sections (60 seconds) at 70°C on a hotplate. After washing with distilled water, fresh acridine orange was applied to the slides (30 seconds) again at 70°C on a hotplate. Following final rinsing in distilled water, the slides were heat dried before mounting in DPX.

2.3.5 Computerised image analysis

Quantification of staining of the experimental specimens was undertaken using a computerised image analysis system [Seescan Analytical Services, Cambridge, UK]. This system comprises: a microscope [ORTHOLUX II, Leitz Wetzler, Germany] mounted with a CCD camera [Sony Inc., Japan], which is connected to a dedicated computer terminal and software.

For immunofluorescence staining, illumination of the specimens was by a UV source emitting at a constant level, in conjunction with separate filter blocks for cyanine-3 (red staining, peak emission wavelength 570nm) and FITC (green staining, peak emission wavelength 520nm). For viewing sections stained with

thionin/acridine orange, a bright light was used, whose current was stabilised to a constant level via a transformer.

The CCD camera allows capture of the section image under view. These images are digitised within the computer and stored on 128MB 90mm optical laser disks [3M, USA]. An automatic pattern recognition programme permits the capture of adjacent images without overlapping. Before analysis, the captured images are edited to improve contrast and facilitate quantification. This involves automatic background subtraction, resulting in the images being further automatically enhanced by reduction of "binary noise". The removal of images less than 3 pixels in size further reduced binary interference while manual editing removed artefacts due to background staining. The image of the staining was finally thresholded, with levels being kept within constant limits for each experiment. The edited image was then quantified according to defined parameters (see below). Following quantification, the data was downloaded onto a 2MB floppy disc [3M, USA] and transferred to a PC for data processing and analysis.

For each animal and each antibody, measurements were made from two sections and the results averaged. Reproducibility was verified by repeat blind analysis of the coded specimen. The measuring parameters used in this project are summarised below.

1. Area of immunostaining

   The amount of axonal regeneration, Schwann cell density, and macrophage infiltration within the nerve/graft complexes was indicated by the measurement of total stained area within the specimens at selected fixed points. These include an area (one screen width wide), 2mm from the proximal anastomosis within the graft tissue (Area G); 2mm distal to the distal anastomosis (Area D); and for sandwich grafts, 2mm within the autograft segment (Area M), all under x25 objective magnification. The total area of staining across the full width of the graft was calculated by summing the measurements from adjacent fields (Figure 2.4). The total area of

staining across the graft can then be expressed as a percentage of the area of nerve that was analysed.

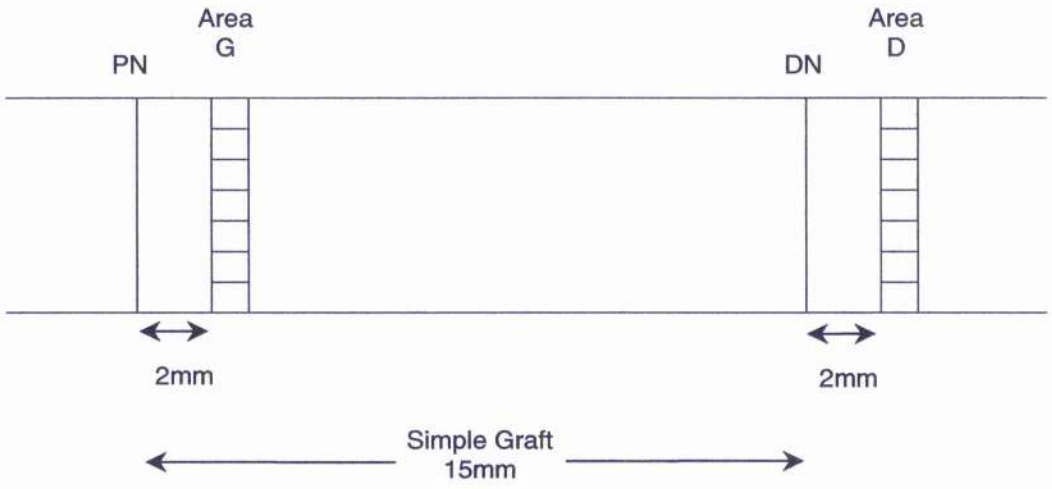
2. Myelinated axon counts

Under x40 objective magnification, three random fields within the posterior tibial nerve were measured for each section, with a minimum of two sections analysed from each nerve. The images were automatically thresholded and manually edited to remove artifacts and non-axonal components of the nerve e.g. blood vessels and Schwann cell bodies. The number of normal fibres was automatically counted from each captured image. The internal and external perimeters of the myelin sheaths were also measured, and the myelin thickness, fibre and axonal diameters calculated from these. The image of the whole posterior tibial nerve was also captured from the semithin transverse sections under x4 objective magnification.

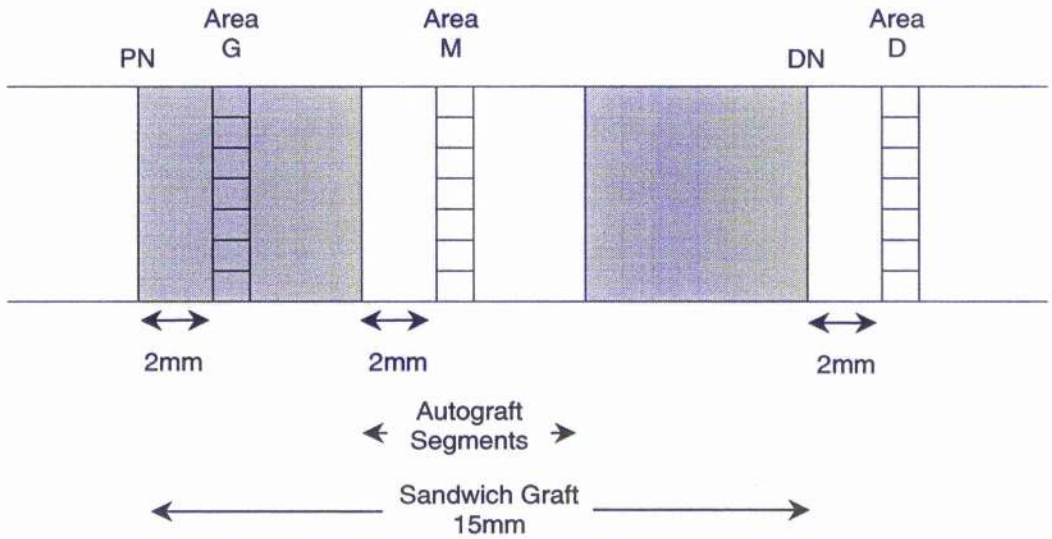
Figure 2.4 Diagram of a longitudinal histological section.

The distances along the nerve/graft complex, measured from the proximal (PN) and distal (DN) neurorrhaphies is given in millimetres (mm). Each small rectangle represents a microscope field. The total area of staining was calculated by summating adjacent fields.

a) For Simple grafts: quantification of staining took place within the graft 2mm from the proximal neurorrhaphy (Area G) and 2mm distal to the distal neurorrhaphy (Area D).



b) For Sandwich Graft: Quantification of staining took place in areas G and D as with simple grafts, with the addition of an area 2mm within the autograft segment of the sandwich graft construct (Area M).



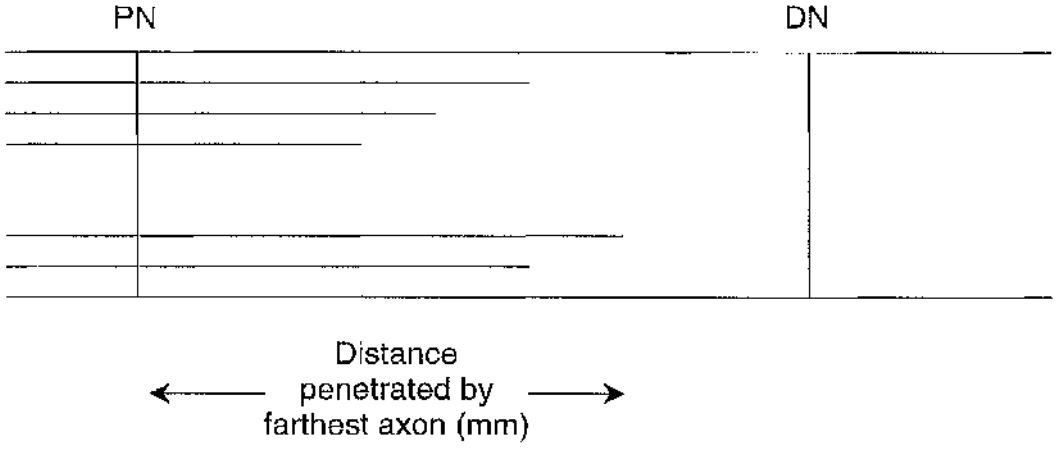
2.3.6 Axonal regeneration distance

This was the distance reached into the graft tissues by regenerating axons from the host proximal nerve. Specimens were inspected for identification of the farthest travelled axon and this penetration distance was measured from the proximal neurorraphy (Figure 2.5) using a calibrated graticule at x10 objective magnification.

The proximal neurorrhaphy was identified by the presence of suture holes and by a small area of randomly orientated axons, on either side of which axons were organised parallel to the long axis of the specimen. In specimens more than fourteen days post surgery, this feature was still present although less distinct.

Figure 2.5 Measurement of axonal regeneration distance

Diagram showing measurement of maximum axonal regeneration distance into the experimental grafts at 7 days (PN = Proximal neurorrhaphy, DN = Distal neurorrhaphy).



2.3.7 Photography

All microscopic images were photographed on an Olympus BH2 microscope mounted with a dedicated Olympus camera and connected to an automatic exposure meter [Olympus Optical Co. Ltd., UK]. Immunofluorescent images were visualised using filter blocks for cyanine-3 and FITC as already mentioned (see Section 2.3.5). Fluorescent UV photography was carried out using Kodak Elite 400 film [Kodak, UK].

Bright light colour photography was carried out with Kodak 160T slide film [Kodak, UK]. Per-operative photographs were taken using a microscope mounted camera and Kodak 160T film as before. Unless otherwise stated, photographs illustrated within this document have been presented in a proximal to distal orientation from left to right.

2.4 STATISTICAL ANALYSIS

Statistical analysis was carried out in collaboration with Mrs. Caroline Dore, Senior Medical Statistician at the Royal Postgraduate Medical School, Hammersmith Hospital, London. Prior to commencement of the experimental work, the minimum number of animals likely to allow a valid statistical outcome (six in each group) was estimated from previous work and following discussion with Mrs. Dore. Commercially available software packages were used to carry out the statistical analyses including STATA [STATA Corporation, Texas USA] and SIGMASTAT [Jandel Scientific, Germany]. The normality assumptions for all data were checked using Shapiro-Francia's W' test, while Bartlett's test was used to check the assumption of equal variances. In some case (detailed in text), logarithmic transformation of data from measurements of area of staining improves the validity of the assumption of equal variance. Other test methods when variances are not equal include assessments based on ranks e.g. Kruskal-Wallis One Way Analysis of Variance (ANOVA). When required, relevant pairwise multiple comparison

procedures were undertaken for each individual analysis as identified in the relevant chapters.

Generally, a three way analysis of variance (ANOVA) was used to compare the results from staining of axons, macrophages and Schwann cells in relation to graft type, presence of immunosuppression, and time course. A further one way ANOVA was used to compare all groups at each time point. Multiple comparison procedures were then used to compare every possible pair of group means at each time point. For each group, the mean and 95% confidence interval were calculated. The confidence interval was the pooled estimate of the standard deviation from the one way ANOVA performed at each time period. If logarithmic transformation had been undertaken then the means and 95% confidence intervals were transformed back to the original scale of measurement for reporting.

All graphical representations throughout this work are of group means presented with standard error bars.

CHAPTER THREE

Early Morphological Features Following Peripheral Nerve Allografting

3.1 INTRODUCTION

Experimentally, long nerve allografts of up to 5cm in rat, rabbit [123,205,211,215] and primate models [129,132,185] have regenerated to a similar degree to autografts when fully immunosuppressed. However, immunosuppression for non-life threatening conditions using drugs capable of life-threatening consequences is still generally perceived to be unethical. Temporary immunosuppression would seem feasible as the regenerating axons are host derived and much experimental time has been devoted to investigating this possibility. Unfortunately, while all investigators agree that a rejection reaction takes place, some note subsequent recovery with ultimate regeneration similar to non-allogeneic controls, [134,185,196,216,262] while others find that subsequent regeneration is not as good and do not feel that temporary immunosuppression has a place in current clinical practice [125,204,247,263] . Despite the advent of hand transplantation opening up the debate, the balance of opinion regarding non-essential allotransplantation is one of caution [234] . This is also expressed in standard textbooks of reconstructive surgery regarding the role of nerve allografts in the future [45] .

With immunological rejection the non-neuronal cells are destroyed [151,221,247,264] , leaving an essentially acellular conduit. Rejection in a peripheral nerve allograft model becomes evident around 4 days post-transplantation, peaking around 9-14 days and lasting up to six weeks, depending on the ferocity of the immunological response [189,209,247,265] . Therefore graft cellularity may not be deleted in a sudden immunological reaction. A more likely staged response might allow enough host Schwann cells to migrate into the rejecting nerve allograft to repopulate them, in the same manner as acellular conduits are repopulated [63,76,151,266] . The more positive results from temporary immunosuppression would suggest that this does occur [134,216,220,267] . However, while many experiments using cyclosporin (CyA) treated nerve allografts concentrate on durations in terms of months with high powered morphological assessments on largely transverse histological sections [97,134,250,268] , more limited data exists as to events occurring within the grafted nerve

in the early regeneration period in immunosuppressed and non-immunosuppressed animals. There is also often a lack of comparative data with suitable control groups [125,204,210,211,216] . Using longitudinal histological sections instead of transverse ones will also allow analysis of regeneration as it progresses through the graft with time. As CyA has been associated with a degree of central neurotoxicity [255,256] it is also important to assess the effects of CyA on standard non-allogenic grafts [269] .

3.2 AIMS

The aims of this experiment were to quantify and assess the pattern and progress of early axonal regeneration within a nerve allograft model during the time period when immunological rejection was most likely to occur. Immunosuppressed and non-immunosuppressed groups were compared to assess their respective regenerative potential.

The effects of any potential genetic disparity were assessed by comparing allogeneic, isogeneic and autogenous nerve grafts. This combination would also provide the opportunity to assess any differences in regeneration potentially attributable to CyA treatment.

3.3 EXPERIMENTAL PROTOCOL

The surgical and anaesthetic techniques employed in this study are described in Section 2.1. Inbred adult Lewis (Lew) rats were the primary host animals and provided their own autografts, and isografts for identical litter mates. Adult Dark Agouti (DA) rats provided the allograft nerves. A 10mm section of sciatic nerve was removed to create a standard nerve defect. This section was then transplanted orthotopically as either an autograft, allograft, or isograft. The autograft group allows comparison with current accepted clinical practice and the isograft group

should produce similar results to the autografts if the animals are a representative inbred population i.e. they show no genetic disparity between individuals. These groups were further divided according to whether or not they were immunosuppressed (Table 3.1). The sole immunosuppressant used was cyclosporin A (CyA). This was delivered subcutaneously in a dose of 5mg/kg/day, and in these experiments was made up from parenteral formulation cyclosporin (25 mg/ml) (Chapter 2.3). The CyA was administered from the day preceding surgery for the complete period of each experiment.

Table 3.1 Experimental Groups (n=6)

Group	Code	CyA	Duration CyA (days)		
Allograft	(Allo+CyA)	yes	7	14	21
Allograft	(Allo-CyA)	no	7	14	21
Autograft	(Auto+CyA)	yes	7	14	21
Autograft	(Auto-CyA)	no	7	14	21
Isograft	(Iso+CyA)	yes	7	14	21
Isograft	(Iso-CyA)	no	7	14	21

Longitudinal nerve/graft specimens were harvested at 7, 14 and 21 days post-operatively, to assess the nerve graft morphology over time. These times corresponded to the duration of CyA administration in each group. Details of the techniques for tissue fixation and staining are given in Chapter 2. Indirect immunofluorescence was utilised to identify the elements under investigation in this experiment which were: Schwann cells, macrophages, vascular endothelium and neural axons. Staining in the graft sections was quantified within an area 2mm into the graft (Chapter 2) as measured from the proximal neurorraphy (termed Area G). This area was one frame wide and extended across the width of the nerve/graft complex. Assessment of axonal penetration into the graft tissue was undertaken at 7 days only and is also described in Chapter 2.

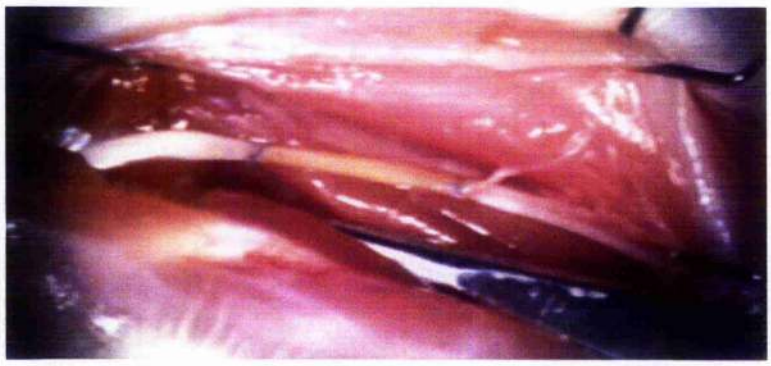
3.4 RESULTS

3.4.1 Macroscopic features

Grafts were inspected on retrieval for differences in colour, texture, integrity, surface vascularity and condition of the anastomoses and wound bed (Figure 3.1).

At 7 days, there was minimal fascial condensation and scarring within the zone of surgery for all groups. Correspondingly, the nerve/graft complexes were easy to remove. All operated nerves were mildly swollen in comparison with unoperated contralateral sciatic nerves. Oedema was especially prominent within all graft segments which were less supple than adjacent host nerve. There were prominent vascular markings running along the epineurial surfaces of the grafts giving them a slightly hyperaemic appearance. These vessels were continuous with host nerve vessels.

Figure 3.1 Macroscopic appearance of a non-immunosuppressed allograft (Allo-CyA) at 21 days



Note the colour difference, which differentiates the allogenic graft from the host nerve proximally and distally (Proximal to the LEFT).

Grafts in all groups, except those of the Allo-CyA group, were similar in appearance to one another. At 7 days, Allo-CyA grafts were yellowish in colour, with a matt, opaque appearance compared to the shiny ivory, more translucent grafts within the other groups. These external features became progressively more evident with time. By 21 days, there was a generalised increase in the degree of fascial adherence and scarring associated with the grafts which was most obvious in the Allo-CyA group. Nevertheless, this did not hamper removal of the specimens. The hyperaemic appearance was reduced in all grafts while still being noticeable within the Allo-CyA group. Here the colour and texture of the grafts remained conspicuously different from the other groups.

3.4.2 Morphological features of axonal regeneration

At day 7, in all groups immunostained regenerating axons were seen crossing the proximal neurorrhaphy. Local to this point the regeneration was haphazard, before orientated axons aligned parallel to the long axis of the graft, running preferentially along the periphery of the graft (Figure 3.2). The maximum distances penetrated through the graft sections were achieved by more advanced axons. These were in advance of the main axonal regeneration front (Section 3.4.3), which generally, at 7 days, was within the proximal third of the graft segments and contained the majority of stained fibres. The non-immunosuppressed allograft (Allo-CyA) group exhibited the poorest regeneration (Figure 3.3). Within the graft, swollen and fragmented axonal remnants were also stained and were a prominent feature in all specimens at this time point. However, it was easy to distinguish the morphology between brightly stained regenerating axons and these dull axonal remnants.

Figure 3.2 Regenerating axons at 7 days

Fig. 3.2a Auto-CyA x 20 (Proximal neurorrhaphy)

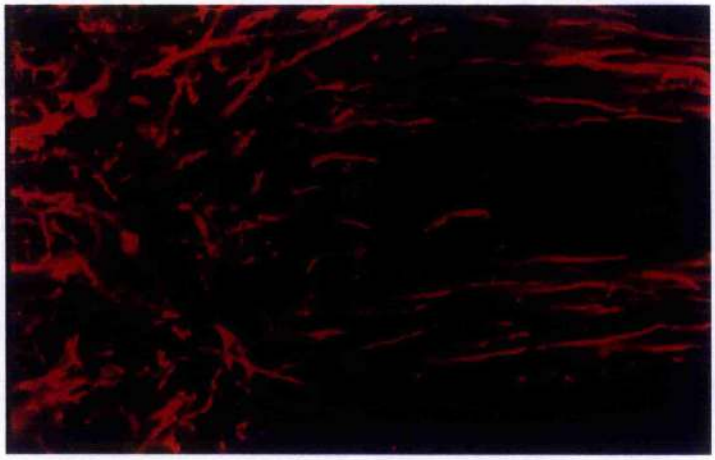
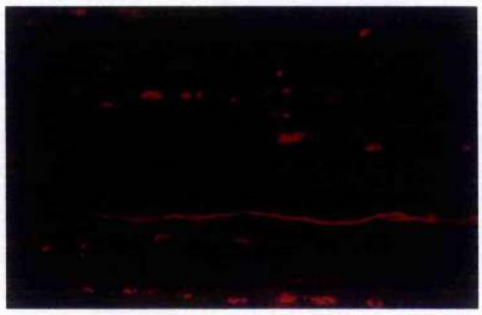


Fig. 3.2b Auto-CyA x 20 (Area G)

PamNF staining of regenerating axons in the autograft group showing: a) the disorganized pattern of axons crossing the proximal neurorrhaphy, then b) having a more parallel and peripheral orientation as the main graft segment is traversed.

Similar morphological features were seen in each group at 14 and 21 days. In particular there were greater amounts of stained parallel, well orientated, fine axons extending across the whole graft width, progressing with time (Figure 3.4). By 14 days a number of axons had reached the distal stump, with the main regeneration front seen to reach the distal third of the graft (Figure 3.5). By 21 days, the main regeneration front had crossed into the distal nerve. However, in the Allo-CyA group there was a time lag so that the 21 day findings were similar to the 14 day findings of the other groups, with a more sparse distribution of axons within the core of the graft and residual stained axonal remnants indicating delayed regeneration.

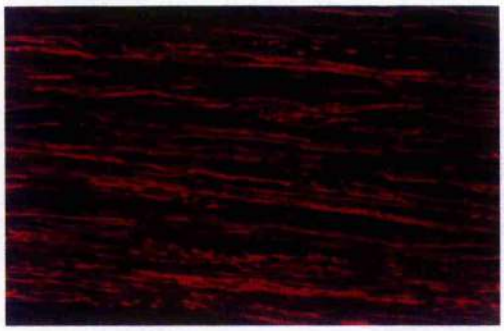
Figure 3.3 Non-immunosuppressed allograft at 7 days



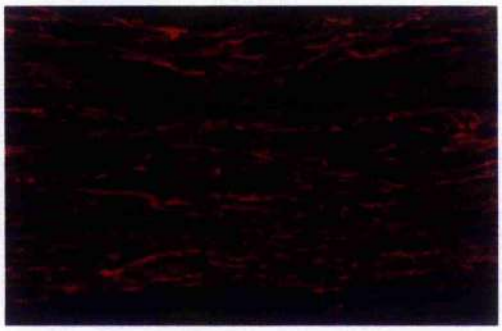
PamNF staining of Allo-CyA x 20 (Area G) showing regenerating axon with axonal remnants

Figure 3.4 PamNF staining of axonal regeneration at 21 days

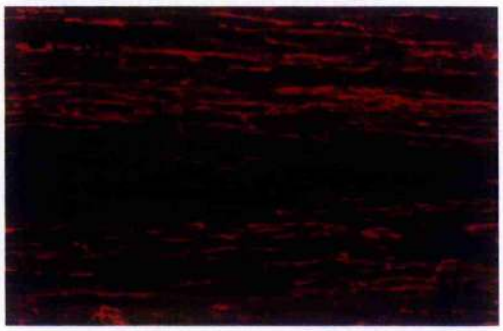
Auto+CyA x 20 (Area G)



Allo+CyA x 20 (Area G)



Allo-CyA x20 (Area G)

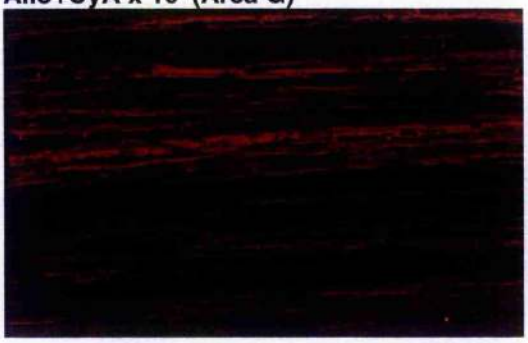


Staining is reduced in the non-immunosuppressed allograft group. Regeneration is similar to that identified in the other groups, following a peripheral and parallel course from proximal to distal.

Figure 3.5 PamNF staining comparing allografts at 14 days

Fig. 3.5a

Allo+CyA x 10 (Area G)

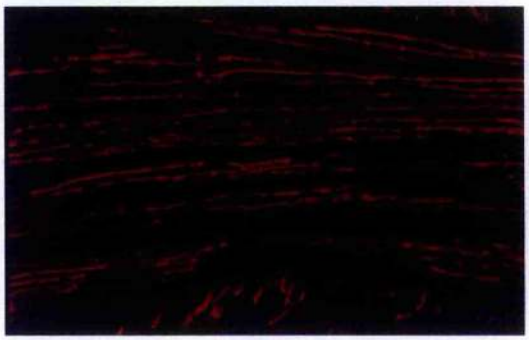


Allo-CyA x 10 (Area G)

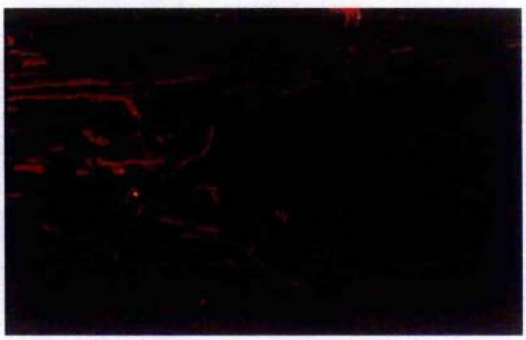
Axons extend across the whole graft width in the Allo+CyA group, but remain peripheral in the Allo-CyA group.

Fig. 3.5b

Allo+CyA x 10 (Distal neurorrhaphy)



Allo-CyA x 10 (Distal neurorrhaphy)



Axons crossing into distal nerve across the whole graft width in the Allo+CyA group, while only a few are reaching it in the Allo-CyA group, and are following a peripheral course there.

3.4.3 Quantification of axonal regeneration

Maximum Axonal penetration Distance

The maximum penetration distance of regenerating axons in each experimental group was measured at 7 days (Table 3.2). At later times, the majority of groups possessed a number of axons that had crossed into the distal nerve, and it was therefore impossible to determine accurately the exact distance of penetration. The non-immunosuppressed autograft group (Auto-CyA) showed the fastest regeneration, with the furthest axons having nearly reached the distal coaptation by

7 days. However, this result was not statistically different from the results obtained from the isograft groups, or the immunosuppressed autograft (Auto+CyA) and allograft groups (Allo+CyA).The non-immunosuppressed allograft group (Allo-CyA) produced the poorest regeneration, which was significantly lower than all the other groups ($p<0.05$).

Table 3.2

Maximum axonal penetration at 7 days (mm) - Mean (+/-SD), n=6

	Allograft	Autograft	Isograft
+CyA	8.62 (+/-3.42)	7.85 (+/-1.03)	8.11 (+/-2.03)
-CyA	4.19 * (+/-0.77)	9.43 (+/-1.10)	8.30 (+/-0.72)

* $p<0.05$ Allograft – CyA vs All other groups
One way ANOVA to compare all 6 groups, Scheffé multiple comparison procedure to compare every pair of group means.

Table 3.3

7 day Percentage area of axonal staining – Proximal Graft Mean (+/- S.D.), n=6

	Allograft	Autograft	Isograft
+CyA	1.31 (+/-0.99)	1.30 (+/-1.11)	0.78 (+/-1.06)
-CyA	0.13 */** (+/-0.25)	4.40 * (+/-4.03)	2.07 ** (+/-1.48)

* $p=0.002$ Allograft – CyA vs Autograft - CyA
** $p=0.025$ Allograft – CyA vs Isograft – CyA

One way ANOVA to compare all 6 groups ($p=0.0009$), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

Proportion of Axonal Staining

An estimate of the amount of axonal regeneration was indicated by the quantification of the total area of neurofilament staining across a constant width of each graft (Area G), which was measured at each time point.

At day 7 (Table 3.3), the Auto-CyA group exhibited the highest level of immunostaining, which was not significantly different from all the other groups, except the Allo-CyA group (p=0.002), confirming the findings from the measurements of axonal penetration. The Allo-CyA group supported less axonal regeneration than the other groups, but because of variation within the immunosuppressed groups it was significantly different only in comparison with other non-immunosuppressed groups.

By 14 days (Table 3.4) the difference in axonal regeneration between the Allo+CyA and the Allo-CyA groups had become significant (p< 0.001).

Table 3.4 14 day Percentage area of axonal staining – Proximal Graft Mean (+/- S.D.), n=6

	Allograft
+CyA	13.86 (+/-4.00)
-CyA	5.80 * (+/-0.86)

* p< 0.001 Allograft – CyA vs Allograft + CyA

One way ANOVA to compare groups (p=0.0004), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

At 21 days post-operatively (Table 3.5), all the groups showed an increased amount of neurofilament staining corroborating the morphological findings. The results from both isograft (Iso+/-CyA) groups and the Auto+/-CyA groups were similar. Axonal regeneration in the Allo+CyA group remained significantly greater than in the Allo-CyA group ($p=0.027$) and was similar to the control groups with or without CyA administration. The Allo-CyA group produced significantly poorer results than the Auto-CyA group and either of the isograft groups ($p<0.002$). These results show that the Allo-CyA group does not support as much regeneration as the other groups, although it does support some regeneration and that this improves over time in a similar manner to the others. Statistical analysis comparing results obtained at 7 and 21 days (Fig.3.5) confirms that axonal staining has increased significantly in all groups ($p<0.004$).

Table 3.5

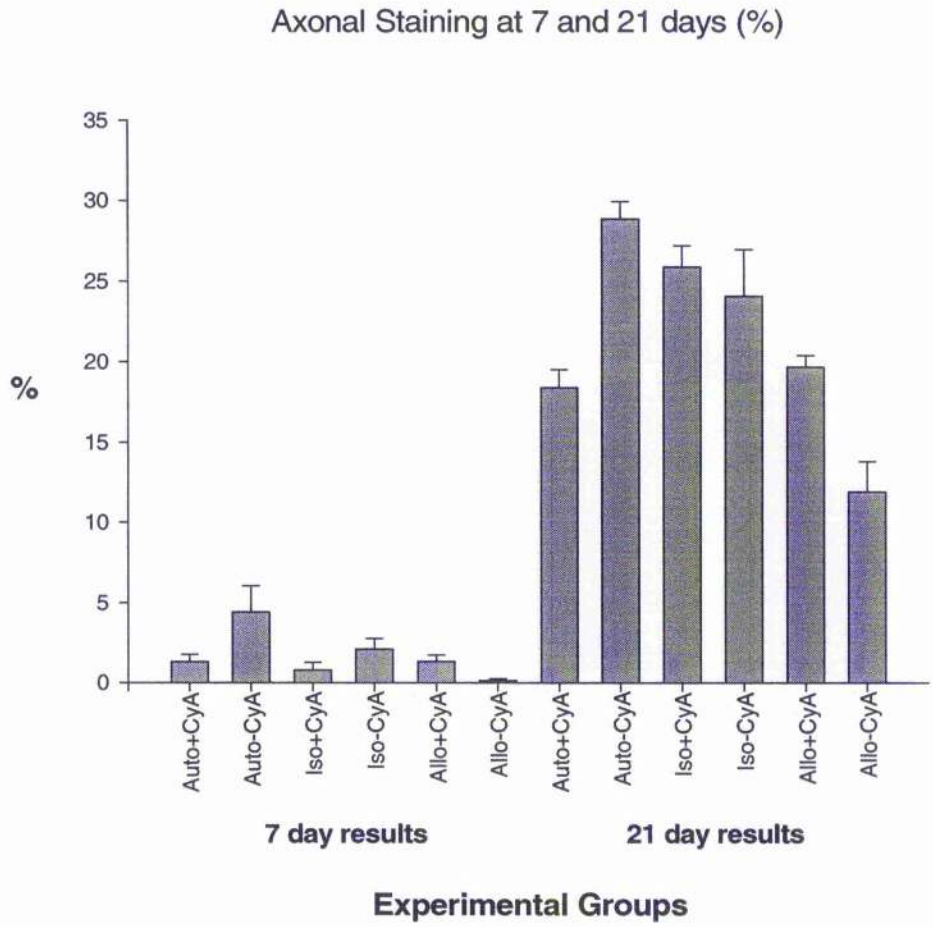
21 day Percentage area of axonal staining – Proximal Graft
Mean (+/- S.D.), n=6

	Allograft	Autograft	Isograft
+CyA	19.66 (+/-1.79)	18.37 (+/-2.78)	25.88 (+/-3.24)
-CyA	11.89 */** (+/-4.66)	28.84 (+/-2.68)	24.07 (+/-7.04)

* p=0.027 Allograft – CyA vs Allograft + CyA
**p<0.002 Allograft – CyA vs Autograft – CyA ; Isograft +/- CyA

One way ANOVA to compare groups (p=0.000), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

Figure 3.6 Comparing axonal staining at 7 and 21 days



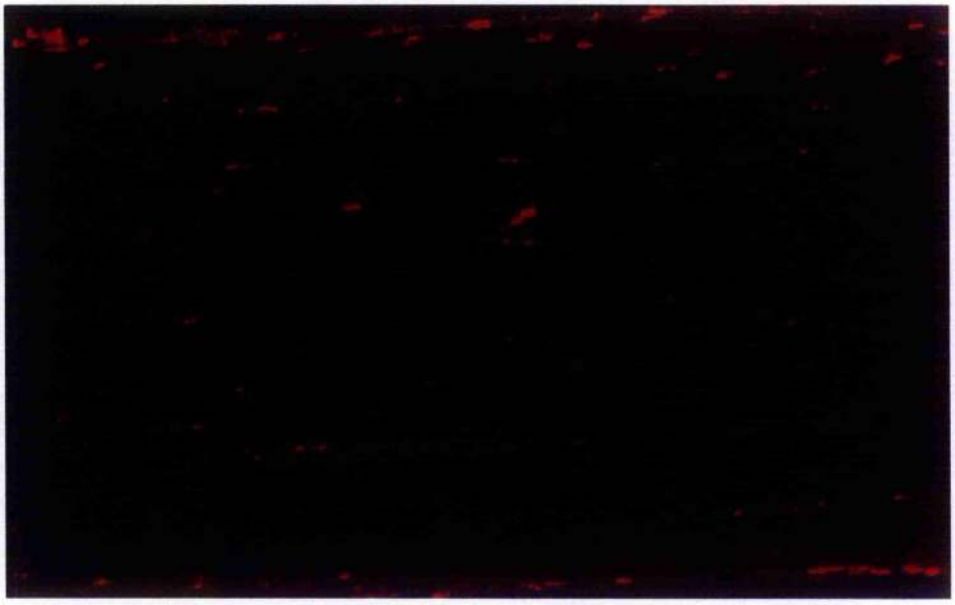
Statistical analysis comparing results in each group between 7 and 21 days showed that regeneration had improved significantly in all groups ($p<0.004$). t-tests were used to compare groups Allo+CyA, Auto-Cya, Auto +Cya. The other groups failed the test for equal variances, therefore the Mann-Whitney Rank Sum test was used.

3.4.4 Morphological assessment of macrophage staining

In all groups, the unaffected proximal nerve furthest away from the proximal neurorrhaphy showed scattered small, ED1 stained macrophages aligned within the nerve structure. These features were identical to those seen in unoperated control nerves (Figure 3.7) where discreet positive staining showed a constitutive population of macrophages. These cells lie along the long axis of the nerve and

have an elongated appearance with dappled cytoplasm and more obvious granular nuclei, with the cell being "squashed" between axonal fascicles within the perineurium. The features noted here are in keeping with the findings of other authors [270].

Figure 3.7 ED1 staining of normal unoperated nerve x 20



Staining of scant constitutive macrophage population

Within the experimental groups, the cell density, size and shape varied considerably from that seen in unoperated sciatic nerve throughout the 21 day period of assessment. The most marked difference was the rise in macrophage density, extending throughout the grafts and into both nerve stumps in all groups. Cell numbers appeared greatest around each neurorrhaphy in all groups. The macrophages were mainly large, bulky cells with granular to foamy cytoplasm containing obvious vesicles and inclusions of debris (Figure 3.8). There were also large numbers of macrophages within the epineurium and in and around blood

vessels. These latter macrophages tended to be smaller, whose morphological features indicated their possible monocyte origins.

The size and features of the macrophages were similar in all groups, except in the Allo-CyA group. Here the macrophages appeared more strongly stained and more extensively distributed than in any other graft groups. The cells appeared clumped together, with exaggerated individual morphological features. Individual cells were difficult to distinguish (Figure 3.9). The large amount of stained macrophages present within the Allo-CyA group did not appreciably alter over the 21 day period, while in all other groups there appeared to be an increase of stained cells over the 21 day period as confirmed by quantification analysis.

Figure 3.8 ED1 staining within graft segments

Fig. 3.8a Auto –CyA x 20

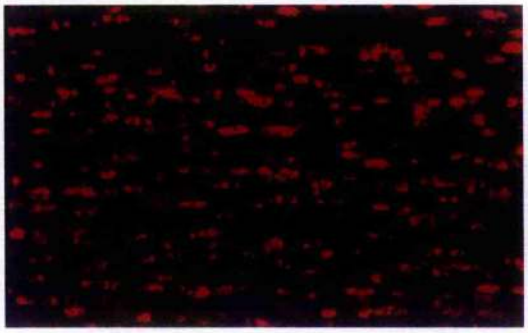
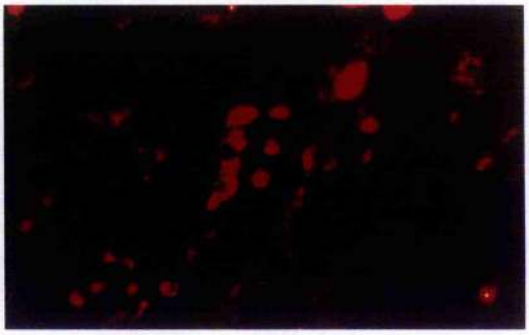


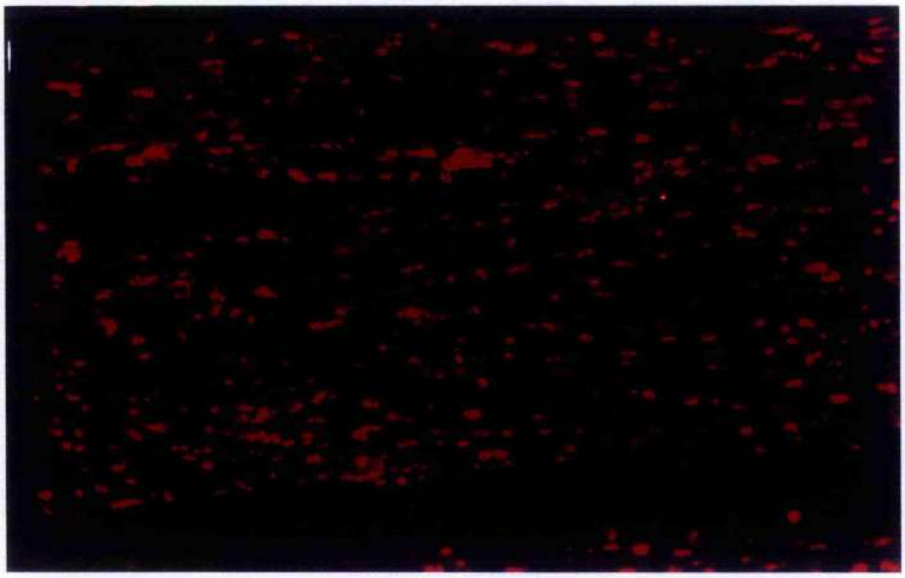
Fig. 3.8b Auto –CyA x 40



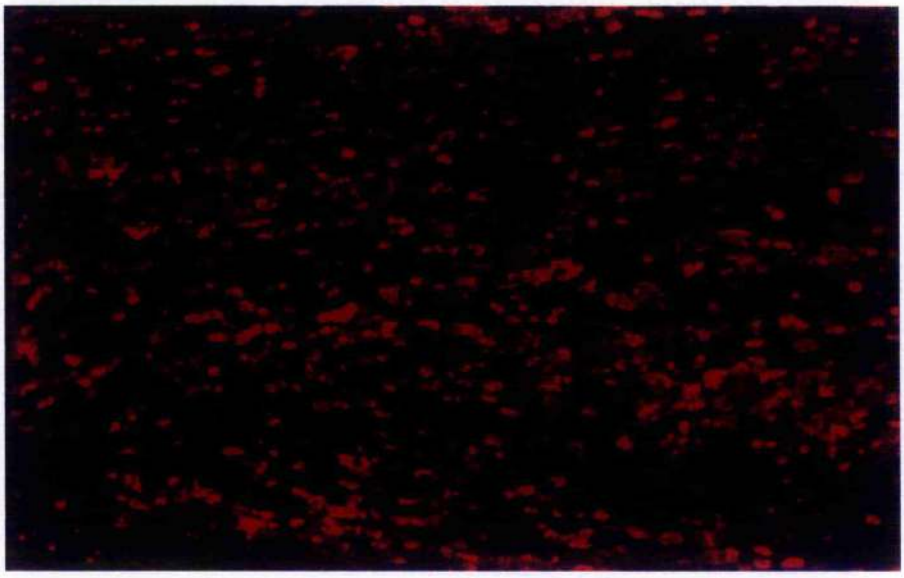
ED1 staining showing the morphological features of macrophages within: a) the graft segment of an Auto-CyA section, and b) identifying small unreactive macrophages within a blood vessel near the distal neurorrhaphy.

Figure 3.9 ED1 staining of allografts at 14 days

Allo+CyA (Area G) x 10



Allo-CyA (Area G) x 10



ED1 staining within allografts sections showing the difference in quantity and features of macrophages. Other non-antigenic groups were similar to the Allo+CyA group.

3.4.5 Quantification of macrophage staining

Macrophage quantification was undertaken within the same Area G of the proximal graft as used for other immunohistochemical staining. Measurement of staining gives a semi-quantitative assessment of the relative amounts of macrophages present within each experimental scenario allowing comparisons to be made between experimental groups [245].

At 7 days (Table 3.6), the Allo-CyA group had significantly greater ED1 staining than all other groups ($p<0.01$), a pattern maintained at 21 days (Table 3.7). There was no significant difference between the Allo+CyA, and isograft or autograft groups regardless of immunosuppression.

Table 3.6

7 day Percentage area of ED1 staining - Proximal graft
Mean (+/-S.D.), n=6

	Allograft	Autograft	Isograft	Normal
+Cya	9.35 (+/-1.99)	11.58 (+/-3.55)	11.87 (+/-3.27)	
-CyA	27.12 * (+/-5.47)	13.27 (+/-3.16)	7.37 (+/-1.82)	0.79 ** (+/-0.27)

* $p< 0.01$ Allograft - CyA vs All other groups
** $p<0.001$ Normal unoperated nerve vs All other groups

One way ANOVA to compare groups ($p=0.000$), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

As there was no statistical difference within either the isograft or autograft paired groups at 7 or 21 days, only the allograft groups were analysed at 14 days (Table 3.7). Here there had been an increase in macrophage staining from 7 days within the Allo+CyA group which was not significant. The amount of staining within the Allo-CyA group remained relatively unchanged, although the results between the two groups were consistently significantly different at all time points ($p<0.004$).

Table 3.7

Percentage area of ED1 staining in Allografts – Proximal Graft Mean (+/-S.D.), n=6

	7 days	14 days	21 days
Allograft + Cya	9.35 * (+/-1.99)	12.80 * (+/-2.81)	17.11 * (+/-4.60)
Allograft - CyA	27.12 (+/-5.47)	27.88 (+/-7.02)	30.26 (+/-2.99)

* $p < 0.004$ Allograft + CyA vs Allograft –CyA at every time point

Two way ANOVA to comparing factors *time* with *CyA*; significant interaction between CyA and time ($p=0.04$). One way ANOVA to compare groups, Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement as represented above.

At 21 days (Table 3.8) there was no within-group difference for the autografts or isografts. Only the Iso+CyA group was significantly lower than the Auto-CyA group ($p<0.04$). Comparisons between groups containing allogeneic grafts at 21 days showed that the Allo-CyA group had the greatest macrophage staining at 30.26% which was significantly greater than the corresponding immunosupressed group ($p=0.022$).

Table 3.8

21 day Percentage area of ED1 staining – Proximal graft
Mean (+/-S.D.), n=6

	Allograft	Autograft	Isograft	Normal
+CyA	17.11 (+/-4.60)	18.95 (+/-6.37)	12.35 ** (+/-2.19)	
-CyA	30.26 * (+/-3.00)	21.30 (+/-2.66)	15.80 (+/-2.39)	0.79 *** (+/-0.27)

* $p \leq 0.05$	Allograft - CyA vs Allograft + CyA, Autograft + CyA, Isograft+/-CyA
** $p < 0.04$	Isograft + CyA vs Autograft – CyA
*** $p < 0.001$	Normal unoperated nerve vs All other groups

One way ANOVA to compare groups (p=0.000), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

Comparison of data within each group at 7 and 21 days (Table 3.9) mirrored morphological findings, showing non-significant increases within the Allo-CyA group but significant changes within the Allo+CyA group ($p<0.005$). The autograft groups and the Iso-CyA group also exhibited significantly increased staining between 7 and 21 days ($p<0.04$) although the Iso+CyA group did not. Generally, in the majority of groups staining levels were lower in the CyA treated groups, although not necessarily significantly.

Table 3.9

7 and 21 day Percentage area of Macrophage staining – Proximal Grafts. Mean (+/-SD), n=6

		+CyA	-CyA
Autograft			
	7 days	11.58 (+/-3.55)	13.27 (+/-3.16)
	21 days	18.95 * (+/-6.73)	21.30 ** (+/-2.66)
Isograft			
	7 days	11.87 (+/-3.27)	7.37 (+/-1.81)
	21 days	12.35 (+/-2.19)	15.80 ** (+/-2.39)
Allograft			
	7 days	9.34 (+/-1.99)	27.12 (+/-5.47)
	21 days	17.11 *** (+/-4.60)	30.26 (+/-3.00)

*p < 0.04 Autograft + CyA between 7 and 21 days
**p < 0.001 Autograft - CyA and Isograft - CyA between 7 and 21 days
***p < 0.005 Allograft + CyA between 7 and 21 days

Comparison of each within-group mean by *t* – test

Figure 3.10 S100 staining of Schwann cells within grafts at 7 days

a)Auto+CyA x 20

b) Allo+CyA x20

c) Allo-CyA x20

Features of Wallerian degeneration are displayed, although note the more irregular and "moth-eaten" appearance of the non-immunosuppressed group suggesting a process in excess of Wallerian degeneration is occurring.

3.4.6 Morphological assessment of Schwann cell staining

All the grafts in these experiments contained constitutive Schwann cells, which underwent the changes of Wallerian degeneration related to nerve injury.

Within the proximal nerves the features exhibited by stained Schwann cells were similar in all groups at all time points; appearing well preserved, tightly packed, and longitudinally orientated with occasional vacuolation. At the proximal neurorrhaphy (PN), thinner elongated immunoreactive Schwann cells appeared to cross into the graft segment in a disorganised manner. A more parallel orientation was resumed as they penetrated further into the graft similar to the findings with neurofilament staining.

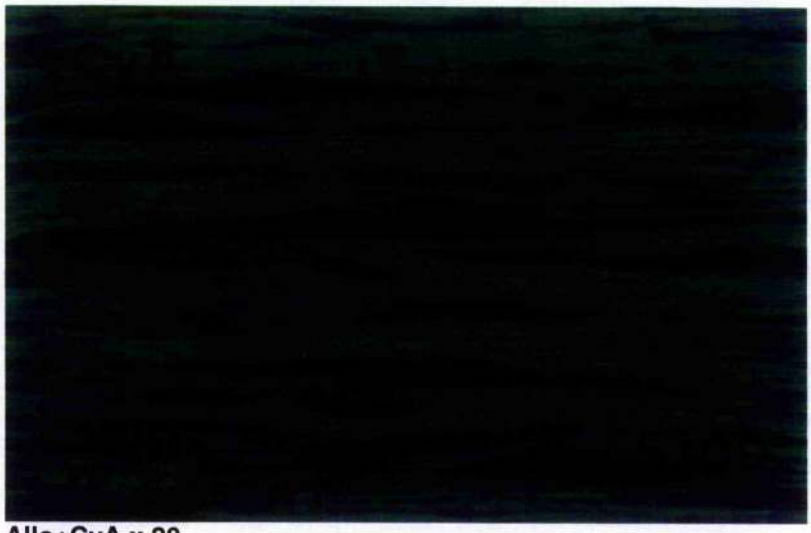
At 7 days (Figure 3.10), within the grafts of all groups, the Schwann cells were large and more rounded in shape with overall parallel orientation, but containing many vacuoles and debris filled vesicles; features typical of Wallerian degeneration. At the distal neurorrhaphy, Schwann cells had similar normal features to those at the proximal neurorrhaphy and also gave the appearance of reaching into the graft (Figure 3.11). By 21 days (Figure 3.12), the grafts of all groups showed a general reduction in degenerating constitutive Schwann cells, with fewer vacuoles, and less marked features than at 7 days. More spindle shaped immunostained cells with more obvious normal features of nuclei and defined orientation forming bands of Büngner were noted within and penetrating into the grafts from both nerve stumps by 21 days.

Figure 3.11 S100 staining of Schwann cell at Distal neurorrhaphy

Auto-CyA x 40

Schwann cell appearing to cross into distal graft from distal nerve

Figure 3.12 S100 staining of Schwann cells in grafts at 21 days



Allo+CyA x 20

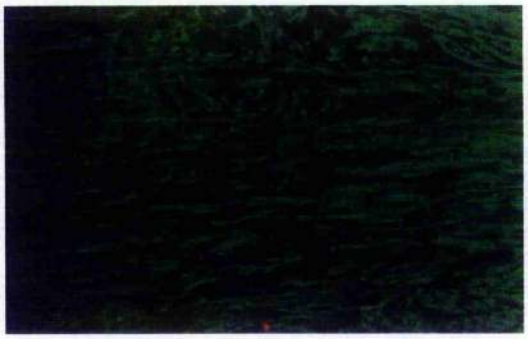
Appearances were similar in all non-allogenic groups (including the immunosuppressed allografts). Schwann cells aligned into bands of Büngner.

In the Allo-CyA group, at 7 days the amount of positive stained cells in the nerve stumps and adjacent graft appeared similar to the other groups. However, by 14 days (Figure 3.13) there was a noticeable reduction in the amount of S100 staining within the graft segment with more evident signs of degeneration. The central portion of the graft was generally the most severely affected with numerous faintly stained, indistinct cells with occasional large vacuolations (Figure 3.13). At 21 days, the relative amounts of normal looking stained cells appeared to have increased within the grafts, with morphological features similar to those from the other groups. These findings are suggestive of a rejection process becoming maximal between 7 to 21 days, and affecting particularly non-immunosuppressed allografted Schwann cells. It also appears that at 21 days there are a significant amount of Schwann cells projecting into the grafts from the distal and proximal host nerves.

Figure 3.13 S100 staining within Allograft groups at 14 days

Allo+CyA

Proximal Neurorrhaphy x 10

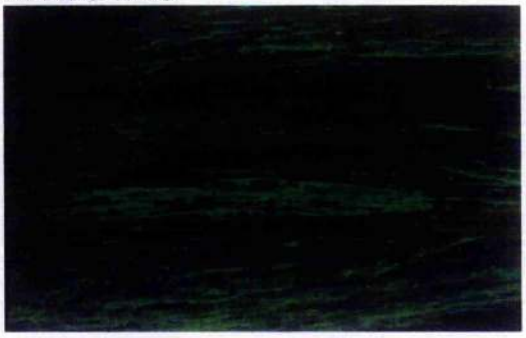


Allo-CyA

Proximal Neurorrhaphy x 10

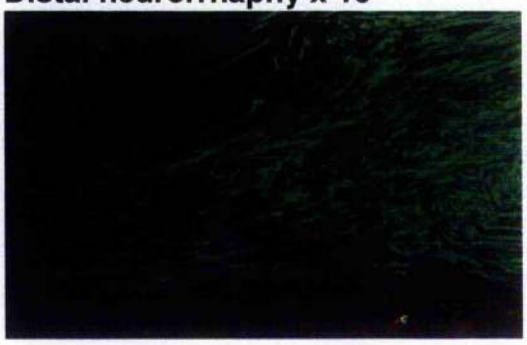
Area G x 20

Area G x 10



Distal neurorrhaphy x 10

Distal neurorrhaphy x 10



Schwann cell density is reduced within the non-immunosuppressed group. Note the pattern of staining is similar to that described in the other groups although more advanced here in the immunosuppressed group, with Schwann cells evident throughout the whole graft structure. Schwann cells appear to be crossing both neurorrhaphies, seen more clearly in the non-immunosuppressed group.

3.4.7 Quantification of Schwann cell staining

As with ED1 staining of macrophages, S100 will stain all Schwann cells regardless of their morphological condition. This means that measurement of the amount of S100 staining will give only an indicative assessment of Schwann cell numbers. The size and features of cells varies throughout the period of Wallerian degeneration and, as the amount of Schwann cell debris was more prominent at 7 days, Schwann cell quantification was only carried out at 14 and 21 days. As allogenic Schwann cells become destroyed by rejection, cell density tended to be reduced in non-immunosuppressed allografts at 14 days, before showing some recovery at 21 days.

Only the allograft groups were quantified at 14 days as the results of morphology and quantification with the other stains has shown the immunosuppressed allograft to be similar to controls, and this was corroborated by the qualitative analysis of Schwann cells. At 14 days (Table 3.10), the amount of S100 stained cells was significantly lower within the Allo-CyA group compared to the Allo+CyA group ($p<0.001$), which confirms the morphological observation.

Table 3.10

14 days Percentage area of Schwann cell staining - Proximal graft
Mean (+/-S.D.), n=6

Allograft + Cya	29.88 (+/-3.90)
Allograft – CyA	8.08 * (+/-2.22)

* $p < 0.001$ Allo + CyA vs Allo - CyA

One Way ANOVA to compare groups. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

Table 3.11

21 day Percentage area of Schwann cell staining – Proximal Grafts Mean (+/-S.D.), n=6

	Allograft	Autograft	Isograft
+CyA	40.92 (+/-9.69)	39.26 (+/-4.99)	38.46 (+/-5.27)
-CyA	8.30 * (+/-4.55)	45.77 (+/-3.77)	47.71 (+/-4.52)

* $p < 0.05$ Allograft – CyA vs All other groups

One Way ANOVA between groups ($p < 0.001$), Tukey test within each group.

At 21 days the results of quantification for the Allo-CyA group remained significantly different from the Allo+CyA group, and were also significantly different from the autograft and isograft control groups regardless of immunosuppression. There was no statistical difference between these controls and the Allo+CyA group (Table 3.11).

3.4.8 Morphological assessment of endothelial cell staining

Von Willebrand's factor (vWF) was used to stain endothelial cells, in order to identify the vascular pattern following grafting and any areas of poor vascularisation and relative ischaemia. At 7 days, multiple small calibre vessels were found around both neurorrhaphies, with more extensive vascularisation within the grafts than in unoperated nerve (Figure 3.14a). Vessels were also seen to penetrate the grafts from the surrounding tissue (Figure 3.14d). Subjectively, the vessels appeared smaller in diameter in the Allo-CyA group than in other groups (Figure 3.14c). From day 14, all groups, except the Allo-CyA group, had clear patterns of moderate to large branching blood vessels traversing the entire grafts (Figure 3.14b). In the Allo-CyA group small calibre vessels persisted within the

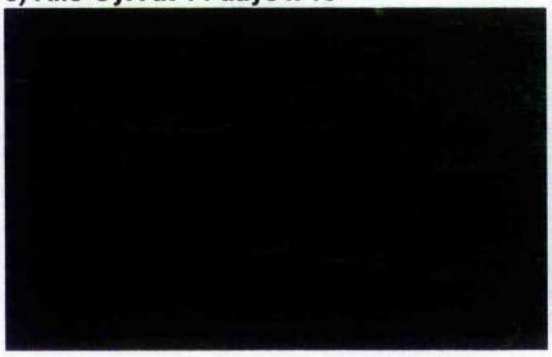
graft and around each neurorrhaphy (Figure 3.14c, 3.14d). By 21 days, blood vessel pattern and density were similar in all experimental groups. These findings suggest that, following grafting, in the Allo-CyA group there is a longer relative ischaemic period than in the other groups, a further contributing factor leading to poorer central core regeneration.

Figure 3.14 Endothelial staining with vWF in Area G

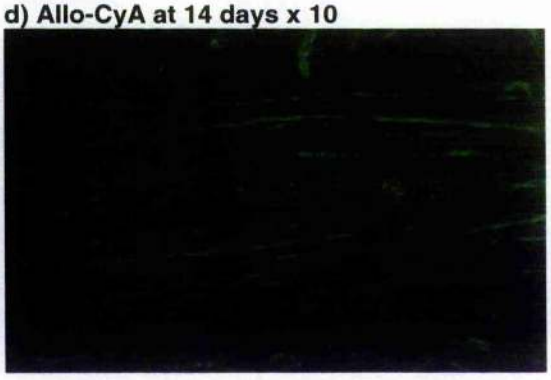
a) Normal nerve x 10

b) Allo+CyA at 21 days x 10

c) Allo-CyA at 14 days x 10



d) Allo-CyA at 14 days x 10



Following graft insertion, multiple small vessels were seen around both neurorrhaphies and within the grafts. By 21 days, staining identified larger caliber, more mature looking vessels, appearing more like those in normal nerve (3.14a, 3.14b). Vessels were less distinct within non-immunosuppressed groups compared to the other groups (3.14b, 3.14c). Similar to axons and Schwann cells, vessels were identified in peripheral sites during earlier time periods. Some vessels were seen penetrating the grafts from the wound bed (3.14d).

3.5 DISCUSSION

This study has investigated the early post-engraftment period comparing both immunosuppressed and non-immunosppressed peripheral nerve allografts using four separate but integrally related features: axonal regeneration, macrophage infiltrate, Schwann cell morphology and vascularisation. By using longitudinal nerve sections events occuring within the proximal and distal host nerve ends can be compared directly to events within the allograft at each time point under investigation. We have shown that axonal regeneration will occur to a limited extent within a nerve allograft without immunosuppression and that its advancement follows the same pattern as its immunosuppressed counterpart and relevant control groups. Rejection of allogenic cells does occur but they seem to be replaced gradually, allowing axonal regeneration to increase slowly.

Cyclosporin has been extensively used in the investigation of peripheral nerve allografting [97] . It has been shown to be an extremely effective single agent immunosuppressant [197,199,202] , and since its discovery in the 1970's it has revolutionised transplantation medicine. it has been shown to have relatively little toxicity in the rat within the time scale of these experiments [206,213,253] . Administration by the subcutaneous route provides good drug bioavailability while being less stressful on the animal models when compared with other possible methods [206,213,254] . We used the parenteral formulation of CyA in the well-documented dose of 5mg/kg/day [206,209] . This dose has been reported to prevent rejection and allow axonal regeneration comparable to autograft and isograft controls [207,216,254] .

Axonal regeneration is widely accepted to occur at an average rate of 1mm per day [41] . Although, depending on the site and local factors, rates of 1.0 - 8.5mm per day have been quoted [3,36] . The results from this study indicate a good rate of regeneration for a proximal mixed nerve, with the maximum Auto-CyA group

axonal regeneration distance reaching 9.43mm from the proximal neurorrhaphy over 7 days. Only the Allo-CyA group produced a maximum penetration distance less than that expected, at 4.19 mm over the same period. However this compares favourably with results extrapolated from experiments where fibronectin, PHB (poly-3-hydroxybutyrate) and frozen thawed muscle conduits were assessed [60,65,73] . Comparing the results of axonal penetration with the features noted morphologically, it is apparent that the most advanced axons in all groups were mainly evident close to the periphery of the grafts. With time, each graft core became filled with regenerating axons.

From the results of macrophage staining all groups exhibited results greater than normal nerve. The constitutive population of macrophages is reported at between 1-4 % [27,271] , which is in keeping with the result for normal nerve quoted here, with similar density of macrophages being noted in rat and human peripheral nerves [270] . The greatest inflammatory infiltrate was associated with the Allo-CyA group. This might contribute to the reduced axonal regeneration seen in this group by providing a degree of physical impedance to the advancing axons similar to that seen in fresh muscle grafts where the basal lamina tubes contain cells and debris [272] , and also because of the associated disruption to nerve architecture [152] . However it also suggests that an inflammatory reaction in excess of that normally seen following nerve injury was occurring. This process is likely to be immunological rejection, with its concomitant destruction of antigenic Schwann cells, resulting in poorer regeneration.

Although acute rejection is most associated with a lymphocyte infiltrate, a mononuclear/macrophage infiltrate is also occurring as both cell lines are intimately involved in the rejection process [145,273,274] . In studies of cardiac and renal allotransplantation the degree of macrophage infiltration has correlated with the occurrence and severity of allograft rejection [275,276] . This occurs within a few days of antigen exposure, once aliorecognition and antigen presentation to

immunocompetent cells has occurred [145,247,263] . In the nerve allograft model rejection appears to become maximal around 14 days post-operatively and to extend for a variable time point thereafter, depending on the antigenic load [209,247,277,278] . These observations are consistent with the massive and sustained macrophage infiltrate reported in these experiments.

Macrophages are integral to Wallerian degeneration [20,22,25,144,279] , which accounts for the infiltrate seen in the other experimental groups. They phagocytose axonal and myelin debris [19,20,27] , recycle cholesterol for myelin resynthesis [173,273] and produce cytokines which promote axonal regeneration directly and indirectly through Schwann cell stimulation to produce neurotrophic factors [25,92,94,280] . Schwann cells also phagocytose debris and can synthesise myelin without recycled components although these processes are prolonged in the absence of macrophages [19,94,173,273] . Macrophages are also one of the major antigen presenting cell in allografts [140,145,146] although Schwann cells can also participate in this in nerve allografts [151,163,168,170,281] .

Schwann cells are the major non-neuronal, endoneural cell type, being nine times more common than fibroblasts [282] , and they are highly immunogenic [102,170,264,281] . They are essential to optimum nerve regeneration, as has been shown by many commentators comparing cellular and acellular conduits [52,62,75,238] , and they have the ability to migrate from both proximal and distal nerve ends into bridging conduits [102,151,245,247] . Although we did not attempt to track the two populations of Schwann cells (host and donor) in these experiments, histological findings suggested that Schwann cell migration was occurring from the host nerve stumps. This was especially evident within the Allo-CyA group at 14 days where the greater cellular degeneration highlighted the presence of Schwann cells extending across the coaptation zone. This would be consistent with evidence from axonal regeneration results and macrophage infiltration indicating that reduction of donor Schwann cell staining was secondary to antigenic cell destruction by rejection,

thereby producing poorer axonal regeneration. However, the maximum rejection phase appeared to be limited, as morphological assessment indicated increased Schwann cell density within the allograft by 21 days with spindle shaped elongated cells noted across the whole graft width. Although this finding was not borne out by the results of Schwann cell quantification, the progressive improvement with time of axonal regeneration, both morphologically and quantitatively, provide additional evidence to indicate that an environment more conducive to axonal regeneration was being established in the Allo-CyA group. It should also be emphasised that in a nerve undergoing Wallerian degeneration a variety of Schwann cell sizes are present depending on the stage of dedifferentiation of the individual cells. For this reason the quantification of S100 staining cannot relate in a direct way to cell numbers but, as shown here, the amount of staining can be used to identify trends and provide additional evidence of events observed morphologically.

Experimental findings for the Allo+CyA group and the control groups were not significantly different. The results of this study would indicate that short-term immunosuppression with CyA prevented rejection of antigenic glial and endoneural cell components. Therefore, antigenic host Schwann cells were undergoing Wallerian degeneration at a similar rater as neighbouring host Schwann cells, hence supporting better host axonal regeneration. Other groups have also identified this. [151] Lassner used staining of Schwann cell MHC expression to identify the allograft cells as being of donor origin, while Midha determined Schwann cell phenotype using a mutant mouse strain in an allograft model lacking myelin basic protein so that host and donor could be differentiated [151,266] .

Restoration of circulation to an avascular graft requires contributions from the wound bed and either cut nerve end [52,156,259,283,284] . Previous work in our laboratory using double immunostaining has shown in an acellular conduit, that regenerating blood vessels from the proximal nerve end travel in association with regenerating axons, and the more correctly orientated the blood vessels, the more efficient the

axonal regeneration [259] . Also improvements in establishing the circulation are accompanied by associated improvements in regeneration [64] . The investigation of the relative contributions of inosculation and neovascularisation involved in nerve grafting is beyond the scope of the experiments reported here, except to say that the topic remains under debate [156] . However, the finding of small calibre vessels at 7 days, becoming larger with time and appearing similar to the pattern of vessel alignment noted in the proximal and distal nerves indicates that an active revascularisation process is occurring and maturing with time. The pattern of Schwann cell and axonal distribution supports this, with both elements preferring peripheral, oxygenated sites instead of the relative ischaemia of the graft core. As the blood supply to the graft becomes more established, so Schwann cells and axons are distributed throughout the grafts. Grochowicz, in a microangiographic study observed a similar pattern of nerve graft revascularisation as identified here, with longitudinal orientated vessels within the graft and smaller calibre vessels around the neurorrhaphies in immunosuppressed allografts and autografts [285] . Findings in the experiments reported here suggest that both inosculation and neovascularisation are involved in revascularisation of these grafts.

Identification of multiple small calibre blood vessels at 3 weeks within the Allo-CyA group suggests a more immature blood supply. Vascular endothelial cells are also highly immunogenic [146,148,286] . As part of the rejection reaction circulation will be disrupted with the prolongation of ischaemia directly contributing to the poorer regeneration seen in the Allo-CyA group, and also indirectly through the effects of ischaemia on Schwann cells.

The general results from isograft and autograft groups were not significantly different from each other, supporting the assumption that within these inbred groups there was no significant genetic disparity between animals. Wallerian degeneration and axonal regeneration were found to proceed in similar manners within the control groups. However the presence of macrophages and the

cytokines they produce, most notably interleukin 1 (IL-1), are beneficial to the efficient progression of Wallerian degeneration and regeneration [22,25,92,144,287] . Cyclosporin is known to exert most of its immunosuppressant effects by inhibiting T lymphocyte production of lymphokines, but by effecting T cells, CyA also has indirect effects on macrophages [22,279] , which if sufficient could affect the progress of Wallerian degeneration. CyA also effects the non specific effector mechanisms of rejection of which the monocyte/macrophage system is integral [274] . In these experiments the administration of CyA has produced a slight, but not significant deleterious effect on the responses of non-allogenic control grafts. Neurotoxicity has been reported with CyA, usually with prolonged usage and being identified by more central effects [255,256] . Within the time frame of these experiments CyA did not appear to hinder axonal regeneration. This is in keeping with studies using CyA and the immunosuppressants FK506 and 15-deoxysperguelin where administration of the immunosuppressant improved axonal regeneration [134,227,228,288] .

In view of the inherent problems associated with autografting and the often suboptimal results produced by this management strategy [41,48,289] , in selected cases peripheral nerve allografting remains an attractive proposition. MacKinnon's group have undertaken reported successful immunosuppressed clinical cadaveric nerve allografts [217,224,225] . The results of this study provides further evidence to suggest that the nerve allograft is a viable alternative and that manipulation of the microenvironment may improve the conditions for nerve regeneration without, or with reduced immunosuppressant requirements.

CHAPTER FOUR

Morphological Assessment of the effects of an Allogenic/Autologous Nerve Sandwich Graft on Axonal Regeneration and Immunosuppressant requirements

4.1 INTRODUCTION

Researchers studying acellular or inorganic conduits have noticed that inclusion of Schwann cells [62,75,76,238,239,290,291] , or neurotrophic factors [4,52,65,241-243] improves axonal regeneration through these conduits. Enhanced regeneration has been achieved using cultured Schwann cells [61,62,77,162,238,240] or portions of nerve interposed between portions of conduit [69,244,245,292,293] . This latter technique has been called "stepping stone" grafting [244] , or "sandwich grafting" [245,292] . This technique introduces extra neurorrhaphies but requires no special facilities or expertise other than the skills of a competent microsurgeon. This technique has not been investigated with nerve allografts.

As seen in previous chapters allografts already possess most of the factors associated with an ideal conduit [52,68,97] , but they also contain Schwann cells. It is possession of these antigenic cellular factors which limit the nerve allograft, as peripheral nerve tissue is not immunologically privileged. All of the components within peripheral nerve express antigenic markers. Basal lamina in studies using acellular nerve appears to have little antigenicity [77,92,158,159,180,186,294-296] , however in an experiment involving submaxillary glands Darcy showed a second-set reaction occurred to basal lamina [160] . A higher degree of antigenicity has been reported for vascular endothelium [146,148,151,297,298] and Schwann cells [102,151,152,162,264] which express the constitutive and inducible M.H.C. (Major histocompatibility complex) class I and II antigens [151,162,167-170,281] .

It would seem rational to hypothesise that if a depot of Schwann cells will repopulate other acellular conduits, then a depot of autologous Schwann cells will repopulate a nerve allograft following rejection. As indicated by other work using the same technique [69,245,292,299] , axonal regeneration within the nerve allograft should be improved by addition of a Schwann cell depot, but peculiar to allogenic grafts, increasing the host cell compiement within the allograft may remove or reduce requirements for immunosuppressants. The sandwich graft construct,

formed from both allogenic and non-allogenic elements would therefore represent a chimeric structure. In other organ systems chimeric states have been found to improve host tolerance to allogenic tissue [105,300-303].

4.2 AIMS

The purpose of this study was, firstly, to investigate the effect of inclusion of a depot of host Schwann cells on the axonal regenerative potential within an allograft model. Secondly, to assess the effect the depot of host Schwann cells had on immunosuppressant requirements by comparing immunosuppressed and non-immunosuppressed groups.

Qualitative and quantitative assessment of regenerating axons and Schwann cells were carried out to determine the relationship of these components within the sandwich graft model. Macrophage and endothelial cell staining were also undertaken to assess the effect of the sandwich allograft on inflammation and vascularity. Further comparisons were made between allogenic sandwich grafts and a non-allogenic control sandwich graft composed of isogenic and autologous nerve, as well as with simple nerve allografts and isografts (see Chapter 3).

4.3 EXPERIMENTAL PROTOCOL

The animal models, surgical and anaesthetic techniques employed in this study are described in Chapter 2.1. The parenteral formulation of cyclosporin A (CyA) was the sole immunosuppressant used (Chapter 2). This was delivered subcutaneously in a dose of 5mg/kg/day, beginning the day preceding surgery and continuing for the 21 day duration of the experiment.

Table 4.1

Experimental Groups

Graft	Host	Donor	CyA	Duration	Number
SandAllo + CyA	Lew	DA/Lew	Yes	21 days	6
SandAllo - CyA	Lew	DA/Lew	No	21 days	6
SandCon - CyA	Lew	Lew/Lew	No	21 days	6

Sandwich allografts (SandAllo) were constructed as per Chapter 2.1.2., and were composed of two 5mm sections of DA sciatic nerve with a central 5mm portion of host Lewis nerve. Similarly, sandwich control (SandCon) grafts comprised two 5mm portions of isogeneic nerve with a central 5mm Lewis autologous segment. For a summary of all the groups involved see Table 4.1. An immunosuppressed control sandwich graft group was not used in this experiment as the findings from non-aliogenic control groups in Chapter 3 had not noted any significant differences between isografts or autografts related to immunosuppression.

Details of techniques for tissue fixation and staining are given in Chapter 2.1.3-2.2.3. The technique of indirect immunofluorescence was employed using the following antisera: Pan-axonal marker of neurofilaments (Pam-NF), S100, ED1 and Von Willebrands factor as per Chapter 3. Morphological assessments for qualitative analysis (Chapter 2.2), along with computer assisted image analysis for quantification of area of staining were utilised (Chapter 2.2.5). Three separate areas were measured to quantify staining area. These were: an area 2mm into the proximal graft as measured from the proximal neurorrhaphy (Area G), an area 2mm into the middle autologous graft segment as measured from its neurorrhaphy

with the proximal graft (Area M), and an area 12mm distal to the proximal neurorraphy (Area D) (Figure 4.1).

4.4 RESULTS

4.4.1 Macroscopic

All of the sandwich grafts used in this experiment had healed well by 21 days, and all were easily removed at harvest. However, there were conspicuous differences noted between the allogenic and autologous graft segments especially within the non-immunosuppressed sandwich graft group. The features were similar to those described for simple grafts in Chapter 3, with oedema of all the graft segments and relative hyperaemia of the epineurial surface of the grafts. These features were much more marked within the allogenic segments of the non-immunosuppressed sandwich allografts, due to their juxtaposition between segments of autologous nerve. The yellow toned colour difference previously noted with simple allografts was also evident within the allogenic sections of the non-immunosuppressed sandwich grafts (Figure 4.2).

Figure 4.1

Diagram of quantification areas G, M, and D within sandwich and simple grafts.

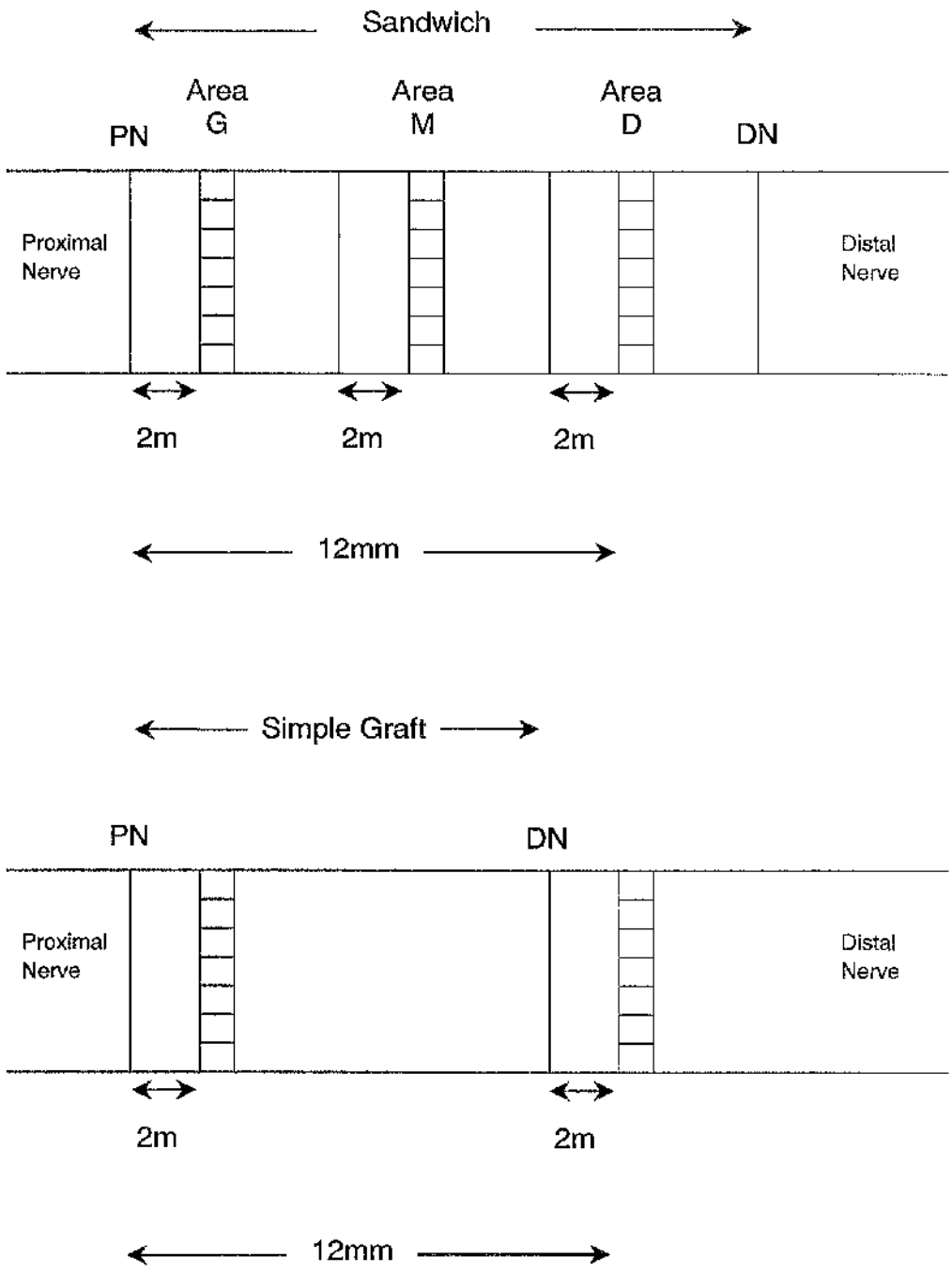


Figure 4.2 Sandwich graft at 21 days

Macroscopic appearance at 21 days of a Sandwich allograft + CyA. Proximal is uppermost.

4.4.2 Morphological features of axonal regeneration

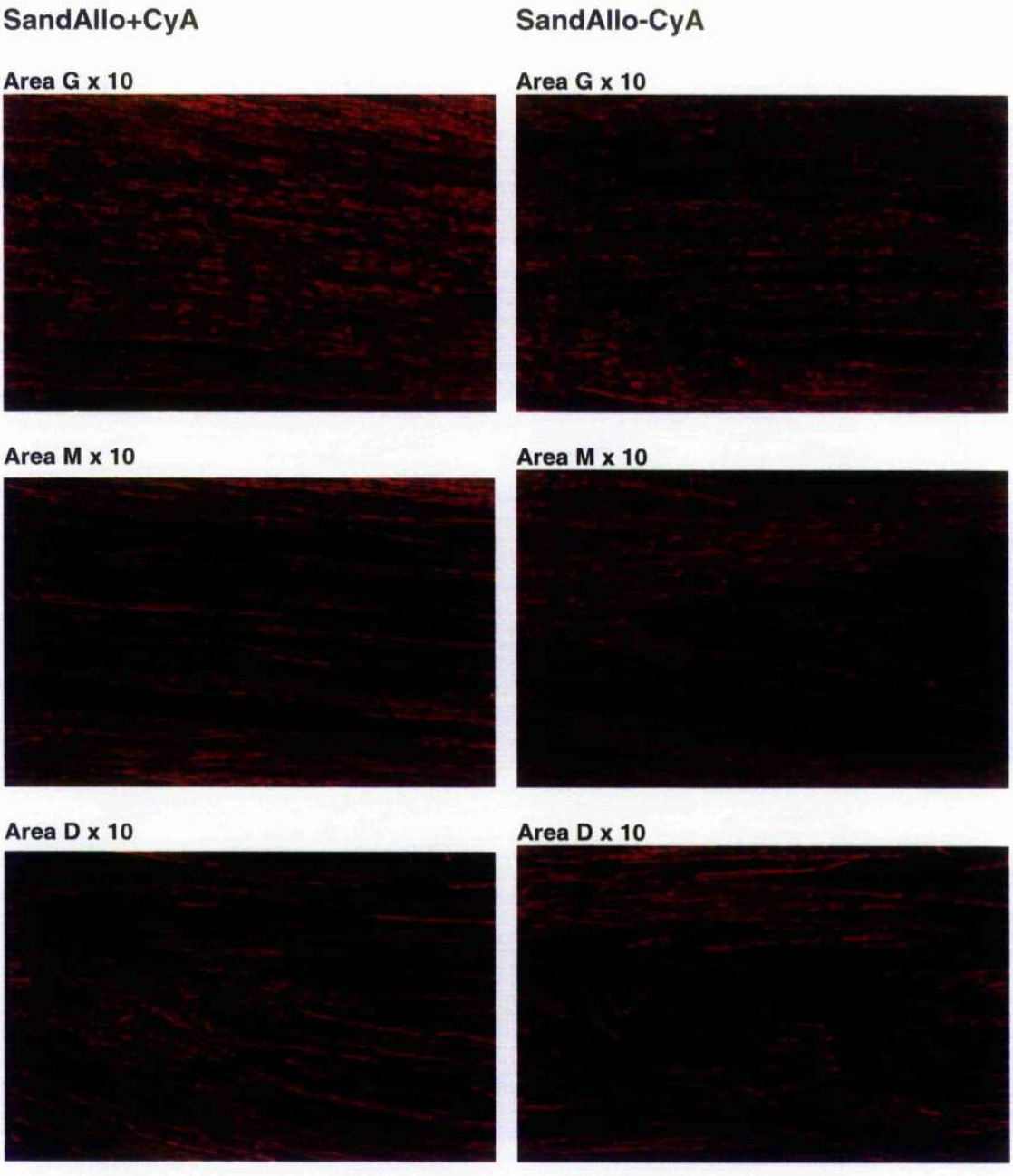
Sandwich grafts were only assessed at 21 days. This time point was noted in experiments comparing simple non-allogenic and allogenic graft models (Chapter 3) to produce axonal regeneration in the former groups across 10mm grafts and into distal nerve. However regeneration within the non-immunosuppressed allograft group exhibited delayed and poorer regeneration, associated with reduced amounts of Schwann cell staining. Based on these observations, expectations of the situation within the sandwich grafts were that by 21 days the peak of immunological rejection would be passed, although probably still ongoing, and allogenic Schwann cell numbers would be maximally depleted. Therefore at this time point, any benefits from the host depot of Schwann cells within the sandwich grafts would be more easily observed. Comparison with the results in Chapter 3

would also identify any gross differences in behaviour between the various types of graft sections employed.

In the sandwich grafts, features of axonal regeneration within the allogenic and non-allogenic graft segments were similar to those described in Chapter 3. The majority of axons had reached the distal graft segment with the most advanced fibres extending into the distal nerves. The sandwich grafts all appeared to have produced regeneration similar to that identified within the non-allogenic grafts in Chapter 3, and certainly much better than the non-imunosuppressed simple allograft group.

The pattern of regeneration again indicated that axons regenerated from an initially preferred course along the periphery of the nerve grafts gradually extending to include the whole width of the nerve graft sections with time. This was indicated by the progressive reduction in the amount of regenerating axons from proximal to distal, and peripheral to central along the sandwich graft structure. Also, at 21 days there was minimal disorganisation of fibres associated with the most proximal neurorrhaphy. However, with successively more distal neurorrhaphies the stained axons crossing the coaptations became more obviously disorganisation and similar to those noted in the early time periods observed within Chapter 3. There was no obvious difference in the amount of axonal regeneration observed within either the immunosuppressed or non-immunosuppressed sandwich allografts, or the sandwich control group (Figure 4.3).

Figure 4.3 Sandwich graft axonal regeneration at 21 days



Pam-NF stained axons showing regeneration as it proceeds from proximal to distal through the sandwich grafts. Features in the control group were similar to those in the Allo+CyA group.

4.4.3 Quantification of axonal regeneration

The sandwich grafts used in this experiment measured 15mm in length and were used to repair a 10mm nerve defect. As qualitative assessment had indicated many axons were present within the distal graft sections, quantification of axonal staining was undertaken within the proximal and distal graft areas (Figure 4.1-Area G and D) at this 21 day time point. The aim of the two measurements was to help establish a more accurate interpretation of regeneration within the different models, especially as theoretically the increased number of coaptations may influence regeneration.

Table 4.2

21 day Percentage area of axonal staining – Sandwich Grafts Mean (+/-SD)

	Proximal graft (Area G)	**Distal graft (Area D)**
SandAllo + CyA	14.94 (+/-1.47)	6.01 (+/-2.87)
SandAllo - CyA	14.84 (+/-2.18)	7.54 (+/-3.16)
SandCon - CyA	10.75 * (+/-2.46)	4.91 (+/-1.13)

Area G
* p<0.05 SandCon vs SandAllo +/- CyA
One way ANOVA comparing groups (p=0.005). All pairwise multiple comparison procedures - Tukey test

Area D
One way ANOVA comparing groups (p=0.233). No significant difference between groups.

Quantification of the sandwich allografts within the proximal allograft segments confirms the findings from morphological assessment (Table 4.2), with no statistical difference identified between the amounts of stained axons present within these

groups regardless of immunosuppression. There was, however, a statistical difference found on comparison with the sandwich control group ($p<0.05$). This difference was not noted when axonal staining was quantified within the distal area of analysis. Here there were no statistical differences noted between any of the sandwich graft groups.

Comparison of quantification within the proximal grafts between the sandwich grafts and the simple grafts from Chapter 3 produces a much more complex picture (Table 4.3). A One Way ANOVA was undertaken on ranks which uses median values for each group, therefore some of the results differ from those given in Chapter 3.

Table 4.3

21 day Percentage area of axonal staining – Proximal Graft Median (25-75%)

	SandAllo	SandCon	Allograft	Isograft
+CyA	14.45* (13.70-16.30)	n/a	19.70** (18.40-20.30)	25.10• (24.30-27.70)
-CyA	14.85* (12.50-17.10)	10.21 (8.61-12.50)	10.55 (9.81-12.80)	23.95•• (22.90-29.50)

* $p<0.05$	SandAllo+/-CyA vs All other groups
** $p<0.05$	Allograft + CyA vs All other groups
• $p<0.05$	Isograft + CyA vs All other groups
•• $p<0.05$	Isograft – CyA vs All other groups

Kruskal-Wallis One Way ANOVA on ranks as data failed equal variance test ($p<0.001$). All pairwise multiple comparison procedures – Student-Newman-Keuls method.

The isograft and immunosuppressed allograft groups have produced the greatest axonal regeneration, with all the sandwich grafts exhibiting statistically poorer regeneration by comparison ($p<0.05$). However, both allogenic sandwich grafts showed significantly better axonal regeneration than the non-immunosupressed simple allograft ($p<0.05$). The results from the sandwich control group were the poorest overall. This was an unexpected result as measurements were taken within an isogenic graft portion and were expected to mirror the results produced by the other isogenic groups. Again, when results from all groups were compared within the distal area of quantification (Area D), no statistical differences were noted (Table 4.4).

Taken together, qualitative and quantitative findings from the proximal and distal assessment areas indicate a potential benefit conferred by the sandwich allograft composition. However, the results from the sandwich control group infer that where no benefit is expected i.e. the graft is not allogenic, then the structure may compromise early regeneration although appearing to even out with time.

Table 4.4

21 day Percentage area of axonal staining – Distal Quantification Area Mean (+/-SD)

	SandAllo	SandCon	Allograft	Isograft
+CyA	6.01 (+/-2.87)	n/a	5.62 (+/-2.61)	4.47 (+/-2.85)
-CyA	7.54 (+/-3.16)	4.91 (+/-1.13)	3.69 (+/-1.49)	6.66 (+/-3.14)

One way ANOVA to compare groups ($p=0.186$). No statistical difference between groups.

4.4.4 Morphological assessment of Schwann cell staining

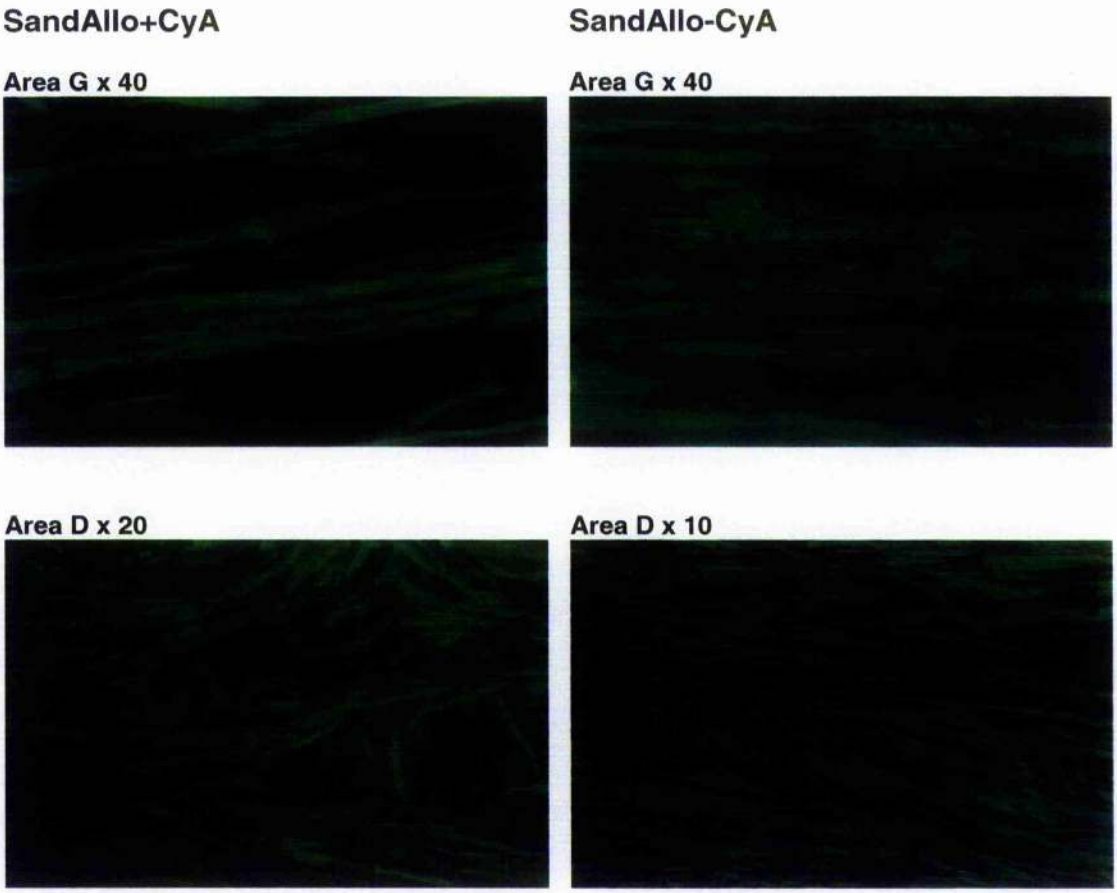
As in the simple grafts (see Chapter 3), Schwann cells within the proximal nerve stumps were well organised, longitudinally orientated and exhibited some vacuolation. Within the sandwich grafts there were less obvious distinctions between any of the groups (Figure 4.4). Generally, there were well orientated longitudinal rows of elongated, normal looking Schwann cells within the grafts and the distal nerve stump. The core areas of the graft sections were where deficient staining, if any, occurred. This was not such a prominent feature as that noted within the simple non-immunosuppressed allograft group presented in Chapter 3. At each of the coaptations, Schwann cells and their processes were seen to extend across these more disorganised zones giving the indication of migration across these areas as noted within the simple grafts. This disorganised appearance was more obvious at the more distal coaptations mirroring the appearance of axonal staining.

4.4.5 Quantification of Schwann cell staining

As mentioned within the experimental protocol, quantification of immunostaining was undertaken within the proximal graft, middle autologous graft and distal grafts of the sandwich grafts: areas G, M and D respectively, (or distal nerve in the case of simple grafts). When comparisons were made with results of similar staining within Chapter 3, only the proximal and distal areas were compared as there was no area corresponding to the central autologous graft within the simple models.

Quantification of Schwann cell staining within the proximal graft sections of sandwich grafts at 21 days indicated that there was significantly less Schwann cell staining within the non-immunosuppressed group compared to the control sandwich graft group ($p<0.05$), but no difference with regard to the immunosuppressed sandwich allograft (Table 4.5).

Figure 4.4 Sandwich graft Schwann cell staining at 21 days



S100 staining of Schwann cells within allogenic sandwich grafts. Schwann cells in both groups are aligning into bands of Büngner. There are some cells still exhibiting vacuolation. Schwann cells are present throughout both graft types, in all areas.

Comparison of these results with the simple graft groups (Table 4.6) shows that all of the sandwich graft groups have produced greater Schwann cell staining than the non-immunosuppressed allograft group ($p<0.05$). This is especially important in the case of the non-immunosuppressed sandwich allograft group.

Table 4.5

21 day Percentage area of Schwann cell staining – Proximal Graft Mean (+/-SD)

	SandAllo	SandCon
+ CyA	31.45 (+/-5.70)	n/a
- CyA	28.04 * (+/-8.62)	41.16 (+/-6.69)

* p<0.05 SandAllo – CyA v SandCon

One Way ANOVA comparing groups (p=0.016). All pairwise multiple comparison procedures – Tukey Test.

Table 4.6

21 day Percentage area of Schwann cell staining – Proximal Graft Mean (+/-SD)

	SandAllo	SandCon	Allograft	Isograft
+CyA	31.45 ** (+/-5.70)	n/a	40.92 (+/-9.69)	38.46 (+/-5.27)
-CyA	28.04 *** (+/-8.62)	41.16 (+/-6.69)	8.30 * (+/-4.55)	47.71 (+/-4.52)

* p<0.05 Allograft – CyA vs All other groups
** p<0.05 SandAllo + CyA vs Isograft –CyA
*** p<0.05 SandAllo – CyA vs Isograft – CyA, SandCon, Allograft + CyA

One Way ANOVA (p≤ 0.001). All pairwise multiple comparison procedures – Tukey Test.

Both of the sandwich allograft groups had significantly poorer staining than non-immunosupressed isograft group, which had the best results. As it was expected, the isograft section measured within the control sandwich graft exhibited similar staining to the simple isografts. Similarly, there was no difference noted within the proximal quantification areas of both the immunosuppressed sandwich and simple

allograft groups. The immunosuppressed simple allograft did however show greater staining than the non-immunosuppressed sandwich allograft but staining was equivalent within both sandwich allograft groups.

Table 4.7

21 day Percentage area of Schwann cell staining – Middle Autograft Section Mean (+/-SD)

	SandAllo	SandCon
+ CyA	28.41 * (+/-5.32)	n/a
- CyA	31.45 (+/-3.42)	38.69 (+/-7.15)

* p=0.02 SandAllo + CyA v SandCon

One Way ANOVA to compare groups (p=0.019), Scheffé multiple comparison procedure to compare every pair of group means. Analysis undertaken on log transformed data to satisfy assumption of homogeneous variances. Data presented in original scale of measurement.

Schwann cell staining within the middle autologous graft section of the sandwich grafts (Table 4.7) identified an unexpected result. No differences were predicted within this area as it was autologous in all groups. However, staining within the immunosuppressed sandwich allograft was significantly poorer than that noted within the control group (p=0.02). There was no significant difference detected between either of the sandwich allograft groups.

Table 4.8

21 day Percentage area of Schwann cell staining – Distal area Mean (+/-SD)

	SandAllo	SandCon
+ CyA	17.59 * (+/-7.64)	n/a
- CyA	31.36 (+/-7.55)	38.02 (+/-11.99)

* $p<0.05$ SandAllo + CyA v SandCon
One Way ANOVA to compare groups ($p=0.005$). All pairwise multiple comparison procedures – Tukey Test.

Regarding the distal area of quantification, another unexpected result was identified within the immunosuppressed sandwich allograft group (Table 4.8). Schwann cell staining in this group was significantly poorer than both the non-immunosuppressed sandwich allograft and control groups ($p<0.05$). This did not agree with the findings of morphological assessment. This finding also affected the results when comparisons were made with the simple graft results (Table 4.9). Here the only group showing significantly different staining from the other groups was the immunosuppressed sandwich allograft group, although it was not different from either the immunosuppressed isograft or non-immunosuppressed sandwich aliograft groups. However, it should be remembered that the quantification area D (12mm from proximal neurorrhaphy in all groups) is within the distal graft segment of the sandwich grafts, while it is within the distal host nerve of the simple grafts. This means that the measurements undertaken from the sandwich allograft groups were the only from allogenic areas.

Generally, these results indicate that the appearance of greater Schwann cell staining within the non-immunosuppressed sandwich allograft group can be attributed to its non-allogenic component.

Table 4.9

21 day Percentage area of Schwann cell staining – Distal Quantification Area Mean (+/-SD)

	Sand.allo	Sand/con	Allograft	Isograft
+CyA	17.59 * (+/-7.64)	n/a	35.10 (+/-9.29)	31.16 (+/-9.29)
-CyA	31.36 (+/-7.55)	38.02 (+/-11.99)	38.46 (+/-6.82)	36.73 (+/-3.20)

* $p<0.05$ SandAllo + CyA vs SandCon, Allograft +/- CyA, Isograft – CyA
One Way ANOVA ($p<0.001$). All pairwise multiple comparison procedures – Tukey Test.

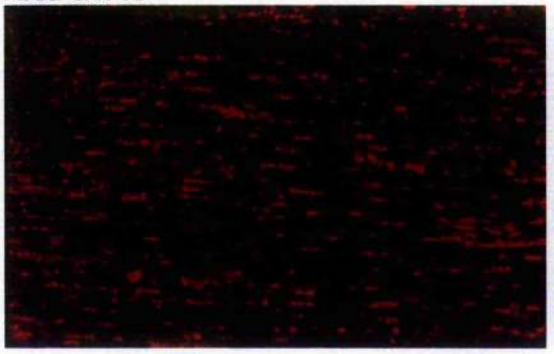
4.4.6 Morphological assessment of macrophage staining

The morphological features exhibited within the sandwich grafts were similar to those described within the simple grafts in Chapter 3. The most exaggerated macrophage forms, with large cells containing vesicles and granular cytoplasm were found within the allograft sections of the non-immunosuppressed sandwich allograft group (Figure 4.5). Features within the autograft sections were similar to the features noted within all other sandwich graft groups. In all groups, smaller ED1 stained cells were found within the epineurium compared to the macrophages within the grafts. This was consistent with findings within the simple grafts. Other consistent features were the increased numbers of stained cells associated with each neurorrhaphy and within the central areas of the non-immunosupressed allograft sections especially.

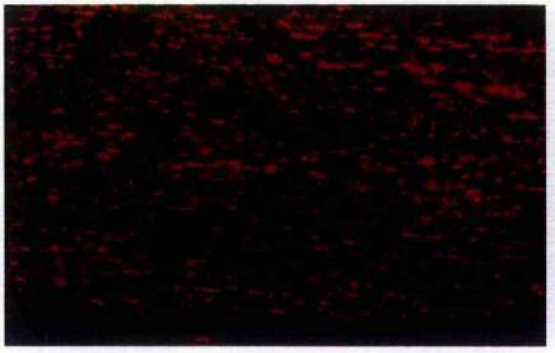
Figure 4.5 Sandwich graft Macrophage staining at 21 days

SandAllo+CyA

Area G x 10

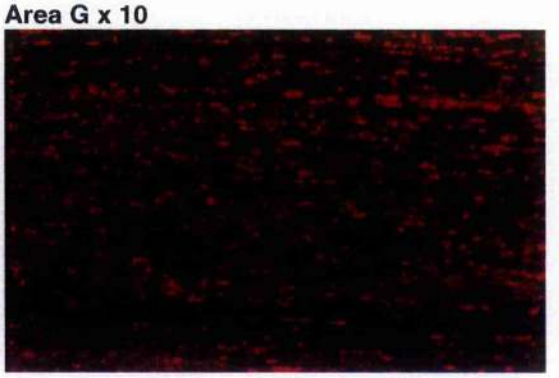


Area M x 10

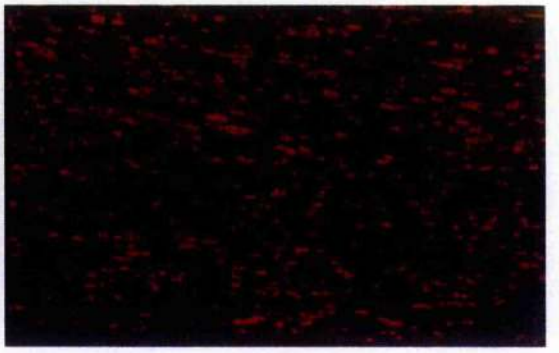


SandAllo-CyA

Area G x 10



Area M x 10



Features of ED1 staining of macrophages in immunosuppressed and non-immunosuppressed sandwich allografts. There are less stained macrophages within the SandAllo-CyA group than were noted within the simple Allo-CyA groups in Chapter 3. The morphological features of the macrophages are also less bizarre. Macrophage staining is similar within the autologous segments of both groups.

4.4.7 Quantification of macrophage staining

Macrophage staining was quantified within the proximal graft, middle autologous section and distal areas of the sandwich grafts, as per Schwann cell quantification.

Not unexpectedly, there were larger amounts of stained macrophages quantified within the proximal allograft section of the non-immunosuppressed sandwich allograft group (Table 4.10) compared to the other sandwich groups (p=0.03). This is consistent with the morphological findings.

Table 4.10

21 day Percentage area of Macrophage staining – Proximal Graft Median (25-75%)

	SandAllo	SandCon
+ CyA	10.70 (10.29-11.06)	n/a
- CyA	17.97 * (16.87-20.14)	10.87 (10.29-13.79)

* p<0.05 SandAllo-CyA vs SandAllo + CyA, SandCon

Kruskal-Wallis One Way ANOVA on ranks as data failed equal variance test (p=0.03). All pairwise multiple comparison procedures – Student-Newman-Keuls method.

When compared to the simple grafts at the same time point (Table 4.11), the simple non-immunosuppressed allografts show the greatest amount of staining. This is significantly greater than the staining within all the other groups. The lowest staining was measured within the immunosuppressed sandwich allograft and isograft groups, and sandwich control group. These were all similar. Staining within the non-immunosuppressed sandwich allograft and isograft groups plus the

immunosuppressed simple allograft groups were also similar of intermediate quantity.

Table 4.11

21 day Percentage area of Macrophage staining – Proximal Graft Median (25-75%)

	SandAllo	SandCon	Allograft	Isograft
+CyA	10.70 (10.29-11.06)	n/a	16.51** (13.04-19.09)	11.65 (11.13-12.21)
-CyA	17.97* (16.87-20.14)	10.87 (10.29-13.79)	30.61* (27.39-32.86)	16.35*** (13.13-18.11)

* p<0.05	Allograft – CyA vs All other groups
** p<0.05	Allograft + CyA vs SandAllo+CyA, SandCon, Isograft+CyA
***p<0.05	Isograft – CyA vs SandAllo+CyA, SandCon, Isograft+CyA
* p<0.05	SandAllo-CyA vs SandAllo+CyA, SandCon, Isograft+CyA

Kruskal-Wallis One Way ANOVA on ranks as data failed normality test (p<0.001). All pairwise multiple comparison procedures: Student-Newman-Keuls method.

Macrophage staining quantified within the middle and distal areas of the sandwich grafts showed no significant differences between sandwich groups within either area (Tables 4.12 and 4.13)

Table 4.12

21 day Percentage area of Macrophage staining - Middle Autograft Section Mean (+/-SD)

	SandAllo	SandCon
+ CyA	11.94 (+/-3.04)	n/a
- CyA	11.12 (+/-2.76)	12.52 (+/-1.36)

One way ANOVA to compare groups (p=0.629). No statistical difference between groups.

Table 4.13

21 day Percentage area of Macrophage staining – Distal Area Mean (+/-SD)

	SandAllo	SandCon
+ CyA	8.13 (+/-2.21)	n/a
- CyA	10.39 (+/-2.34)	8.52 (+/-1.47)

One way ANOVA to compare groups (p=0.157). No statistical difference between groups.

When compared to the other simple graft groups, results of macrophage staining within the distal quantification area show that only the simple non-immunosuppressed allograft group features significantly greater staining than all of the other groups (Table 4.14). These results of macrophage quantification indicate that the sandwich allograft construct is associated with less inflammation than a simple allograft model containing an equal antigenic load.

4.4.8 Morphological assessment of endothelial cell staining

Features of Von Willebrands factor staining of endothelial cells were similar to the findings in Chapter 3. Smaller calibre vessels were associated with the areas around the neurorrhaphies in all groups and also within the allograft sections of the non-immunosuppressed sandwich allograft group (Figure 4.6). More mature vascular patterns were noted within the segments forming the other groups. Although the relatively small size of each individual graft segment making up the sandwich grafts meant that the distinctions between regions such as the neurorrhaphy and the graft cores was not always as clear as within the simple graft groups.

Table 4.14

21 day Percentage area of Macrophage staining – Distal Quantification Area Median (25-75%)

	SandAllo	SandCon	Allograft	Isograft
+CyA	8.63 (6.40-9.32)	n/a	15.93 (8.56-21.83)	8.44 (5.77-11.09)
-CyA	11.13 (9.16-11.76)	8.70 (7.01-8.92)	16.54* (15.10-17.40)	9.98 (9.45-11.20)

* $p<0.05$ Allograft – CyA vs All other groups

Kruskal-Wallis One Way ANOVA on ranks as data failed equal variance test ($p=0.005$). All pairwise multiple comparison procedures – Student-Newman-Keuls method.

Figure 4.6 Sandwich Allograft –CyA Endothelial cell staining

Area G x 10 staining with vWF showing multiple small calibre vessels within the allograft.

4.5 DISCUSSION

The best regeneration possible following nerve transection is when the two cut ends are coapted quickly, accurately, and without non-physiological tension [4,16,46,48,87,88,304] . This scenario however does not guarantee perfect restoration of function even in distal single modality nerves [11,41,48] . If any degree of nerve structure is lost, unless very small and undertaken before structural inherent elasticity is lost, repair will require the use of a nerve graft. Autologous nerve grafting has been established as the best available technique for restoration of continuity across a nerve gap since Seddon popularised it over fifty years ago [80,81] .

The benefits of autografting sit uncomfortably with the detrimental effects of its use. There are limited amounts of expendable nerve for this purpose, and with it, sacrifice of a normal functioning nerve is inevitable. The morbidity associated with the use of autologous nerve grafts is well documented [41,52,289,305] and not

inconsequential, especially when the result is two imperfectly functioning nerve territories. This, along with the desire for a more surgically efficient alternative has fuelled research into developing an ideal "off-the-shelf" conduit.

The single feature shared by all synthetic conduits is the absence of endoneural cells, and Schwann cells in particular. Schwann cells are crucial to nerve regeneration [31] . Although Schwann cells will migrate and survive without axons, the converse is less true [32,75,181,242] , and mitogens inhibiting Schwann cell proliferation will also negatively influence axonal regeneration [76,181] . Bunge has remarked on the broad repertoire of functions Schwann cells have in promoting axonal regeneration [33] . This includes the production of neurotrophic factors [24,40,89,92-94,306] , while providing an ideal substrate for axonal elongation, even in the form of their basal lamina alone, and connection with adhesion molecules [33,39,181,307,308] . Researchers have endeavoured to mimic this by adding Schwann cell cultures [61,62,77,238,309] and various neurotrophic factors [4,52,65,241-243] to acellular conduit materials. While these may have improved regeneration they are some way from clinical practice and technically demanding or difficult to maintain.

The use of a depot of Schwann cells in the form of a sandwich graft within an acellular conduit has been shown to improve regeneration within conduits of vein [69,293] , silicone [244] and muscle [245,292] . The ultimate aim of these studies was to try to improve regeneration over larger defects. In the rat, regeneration is compromised with defects greater than 10mm [4,55,72,245,292,310] .

Calder successfully bridged a 1.5cm defect in rat sciatic nerve using an acellular muscle/nerve sandwich graft [292] . Whitworth extended the technique in a rabbit model using 3 nerve sandwiches between acellular muscle grafts to bridge a 5cm defect [245] . Results from both experiments show regeneration similar to autograft or isograft controls, and superior to muscle grafts alone. Smahel also noted improved

regeneration following the insertion of a 2mm nerve segment within a 14mm vein graft in rats, and using three nerve segments within 40-45mm vein grafts in rabbits [69,311] . However Maeda, although finding superior regeneration within a sandwich graft of nerve and silicone conduit compared to an equivalent sized simple silicone conduit (18mm) noted regeneration to be inferior to isograft controls [244] . This difference in outcomes can be explained by the fact that Calder and Whitworth used a basal lamina conduit of acellular muscle, while Maeda used an inert conduit. Organic conduits of nerve and muscle are more suitable substrates for axonal regeneration than silicone as they allow more efficient revascularisation, orientation and support by way of adhesion molecules [52,72,73,312,313] . Maeda also found that regeneration was poorer in a 24mm double sandwich graft of three silicone conduits and two nerve segments. Orientation of the tiny nerve segments may have been difficult in this model, plus their were 5/10 infection or extrusions associated with the model and 2/10 in the 24mm group failed to regenerate at all [244] . It is unclear whether these were taken into account when statistically analysing his results.

As allogenic Schwann cells are known to be destroyed during immunological rejection [151,156,162,221,314] , it is therefore hypothesised that restoration of the Schwann cell population will improve axonal regeneration similar to that described in other experimental sandwich grafts. Also, replacing allogenic Schwann cells with autologous ones will decrease or halt further rejection removing or reducing the requirements for immunosuppression.

In the experiments described here, a sandwich allograft model has been fashioned along with a composite isogenic/autogenic control sandwich group. The autologous segments were obtained from the nerve resected to form the 1cm defect to be repaired. It should be pointed out here that the nerve defect is not necessarily the same as a nerve gap. Millesi has stressed the difference of these two factors especially as they can lead to confusion with interpretation and comparison of

studies [87] . Nerves are elastic therefore resection of a measurable amount of nerve, or a simple division will produce a larger gap than the defect, while the resected segment appears to shrink. The amount of recoil is individual and position dependant. Therefore, a nerve graft has to be sufficient to overcome the defect and any additional loss of elasticity and non-physiological tension which may have occurred with injury and fibrosis. The size of the graft then represents the gap between the two transected nerve ends. Therefore, the only reproducible parameters when comparing models are the grafts and resection sizes.

Sandwich allografts have been shown here to support axonal regeneration. The pattern of regeneration was similar to that described within simple graft models and morphologically appeared to be as successful as the non-allogenic simple grafts. Axonal regeneration in peripheral graft areas was preferred to the central zones initially. In the sandwich grafts these features were more conspicuous in the distal graft segments, while in the proximal areas excellent regeneration was visible across the whole graft including good axonal organisation across the neurorrhaphies. Axonal remnants and suggestions of axon oedema were not features identified within any of the sandwich groups. As in the other reported examples of sandwich grafting, comparisons with simple conduits of the parent material showed that regeneration was improved by the addition of a nerve segment [244,245,292] . However, important for this experiment was that regeneration was improved regardless of immunosuppression in the sandwich allograft and was superior to that seen within the simple allograft. Regeneration progresses from proximal to distal, therefore at this early time point less regeneration was measured within the distal area where no statistical differences were noted between all groups. These axons represent some of the most efficient to regenerate such that conditions can be assumed to be ideal in their locale. However, the variability of result proximally is a reminder that regeneration within these models is still in evolution, and a final analysis depends on longer term studies.

The sandwich control group exhibited the poorest regeneration overall. This was not predicted as the particular area of quantification was in an isograft segment and therefore regeneration closer to that measured in the simple isografts was expected. There may be several reasons for this but no comparison can be made with available literature as no other author has included an equivalent control group in their experiments [244,245,292] . The increased number of neurorrhaphies would be expected as potential areas limiting regeneration. However, it has generally been found that improving regeneration from that seen in the acellular parent graft has outweighed any deficiencies associated with the suture lines [244,245,292] . As no benefit would be expected from a composite non-allogenic graft such as the sandwich control graft, the deficient regeneration in this case may have been influenced by the increased scarring [86,88] . The other potential source of irregularities within the results may be due to the technical constraints. The length of the graft complexes and their limited flexibility, meant that it was sometimes difficult to get all areas lying flat enough to ensure longitudinal sections were cut at the same depth through the long axis of the sections. While an arrangement of serial cutting was devised to ensure each staining batch contained representative sections from the widest portion of the graft (Chapter 2) it would not have been enough to ensure equal section depths in all areas. This is another reason why multiple areas were quantified. However, the similarity of results measured within the distal quantification areas of all grafts indicates that overall regeneration was not inhibited. Longer term studies are required to fully evaluate this issue.

If the hypothesis that supplying host Schwann cells will improve regeneration in an allograft similar to that experienced within acellular grafts is true, then an improvement in Schwann cell staining would be expected within the non-immunosuppressed sandwich allograft. This was indeed the case. When compared to simple non-immunosuppressed allografts, all groups including the non-immunosuppressed sandwich allograft has significantly greater amounts of Schwann cell staining. The results were comparable between sandwich allografts, and the immunosuppressed sandwich allograft was similar to its equivalent simple

allograft. The results within the sandwich allografts were less than that within the isograft groups which mirrors the findings from axonal staining, however it should be remembered that proximally the area of quantification was within an allogenic segment for the allografts and an non-allogenic segment for the isografts such that a difference would be anticipated.

The standard deviation values show that there was some variability within the groups regarding the quantification of Schwann cell staining. This and the reasons already suggested for variable results may have influenced the lower values measured for the immunosuppressed sandwich allograft within the middle and distal grafts. The middle graft is autologous therefore all groups were expected to be similar. Within the distal quantification area measurements were taken within allogenic segments of the sandwich allografts and non-allogenic segments within all the other groups. The poorer results within the immunosuppressed sandwich allograft group may be spurious and related to group size and the technical considerations given earlier, as axonal regeneration was similar between groups within this area.

Schwann cells are known to migrate from transected nerve into conduits [33,63,102,151,252,277] . They will migrate further in environments more conducive to their survival and function. Therefore they migrate more effectively from the proximal nerve stump in association with axons, as interactions between these two cells are synergystic [33,63,242,252] . Calder noted proximal Schwann cell migration to be double that of distal migration [292] , while Whitworth noted a greater improvement in distal migration following addition of a sandwich autograft graft within a muscle conduit [245] . Schwann cells in both of these studies were also noted to migrate from the sandwich host depot. However, as Schwann cells have a limit to their capacity to repopulate acellular structures from adjacent nerve, the distance between nerve segments was important for regeneration. The use of acellular conduits, because they are devoid of cells makes it easier to attribute the presence of any cells found

within them to the adjacent structures. Serial analyses at early time points can track Schwann cells from the host nerve portions. The situation within nerve allografts where reduction and subsequent improvement in Schwann cell staining, along with the morphological features identified would also indicate that host Schwann cells were replacing the destroyed allogenic Schwann cells in a similar manner. In Whitworth's study using 2.2cm sandwich muscle grafts, at 20 days the whole structure contained Schwann cells [315] . The sandwich grafts here appeared well populated with Schwann cells at 21 days in a similar rodent model.

The technology now exists to label Schwann cells genetically [162] , or to identify cell origin as host or donor based on their MHC [151] or sex chromosomes [238] . These experiments have confirmed in acellular conduits that allogenic donor cells are rejected and host Schwann cells are responsible for conduit repopulation [151,162] . Atchabahian confirmed this by producing rejection when a host repopulated conduit was transplanted back into the original donor [316] .

No immunological tests for rejection were undertaken in this experiment. Findings in Chapter 3 for the non-immunosuppressed allograft with poor axonal regeneration associated with reduced Schwann cell staining and increased macrophage staining indicate that an immunological destruction of Schwann cells was most likely taking place. Schwann cells have been identified along with vascular endothelium as the most significantly allogenic endoneurial cells [102,148,151,152,162,264,297,298] . It is their destruction, along with graft ischaemia which delays regeneration within nerve allografts [61,156,162,264,284] . This delay was noted within the progression of regeneration described in Chapter 3. Delay in establishing end organ/neuronal connections can have profound implications for restoring nerve function [3,16,27,289,317,318] , therefore methods to shorten the delay period are to be encouraged. The longer the graft to be bridged, the longer the delay, and with acellular conduits the less likely that Schwann cells will repopulate the whole graft seriously compromising axonal regeneration [290,310,319,320] . The provision of

additional Schwann cell depots with their neurotrophic benefits will therefore help reduce any delay period. Morphologically, this was suggested by the results of this experiment, as regeneration was similar within the distal graft and distal nerve in the sandwich groups compared to the simple grafts.

Histologically, the pattern of macrophage staining was similar to that within simple grafts. Comparing sandwich grafts, morphologically there was more intense staining and more exaggerated macrophage features within the non-immunosuppressed sandwich allografts. This was borne out by the findings of quantification where there was greater macrophage staining within the proximal graft sections. However, comparison with the non-immunosuppressed simple allografts showed that macrophage staining was significantly reduced. This simple allograft produced the greatest staining proximally. As mentioned in Chapter 3, the addition of CyA would be expected to influence macrophage recruitment indirectly through T-cell effects as both cells have many interactions in cell mediated immunity [168,263,274,279] . The higher levels of macrophage staining mirror the reduced levels of Schwann cell staining in the non-immunosuppressed alloenic groups indicating a likely association between these features. The fact that levels are not as high within the non-immunosuppressed sandwich allograft indicates the reduced allogenicity of this structure as it becomes populated with host derived axons and Schwann cells, as has been hypothesised.

Not surprisingly the middle autologous segment showed no significant difference regarding macrophage staining within the sandwich grafts. The picture within the distal grafts was similar despite the allogenic nature of this segment in the sandwich allografts. Again this may represent efficient graft rejection and repopulation with host cells. Calder found Schwann cell migration from an autologous nerve segment within a muscle sandwich graft to be equal to the rate of migration from distal nerve [292] . Comparison between all groups in the distal quantification area show that only the non-immunosuppressed allograft had

significantly greater staining than the other groups. This measurement was taken within the host distal nerve, 2mm from the adjacent allogenic segment. However, from the findings in Chapter 3, the appearance of this particular group following ED1 staining showed a structure overwhelmed with stained cells, therefore this quantification value is consistent with the morphological findings.

Staining of endothelium confirmed the presence of a vascular structure within the sandwich grafts. Generally the features were similar to those found within Chapter 3 although there were more small vessels related to the increased neurorrhaphies. The non-immunosuppressed sandwich allograft, like its simple counterpart, showed larger amounts of small calibre vessels especially within the allogenic sections. This may represent a more immature system developing in the face of a rejection process. As in Chapter 3, regeneration and Schwann cell repopulation were likely to be poorest, if identified, within the graft cores. Although there were no areas identified as devoid of blood vessels within the sandwich grafts, unlike within the simple non-immunosuppressed allograft, the delayed activity within the graft cores may represent a combination of later revascularisation than the periphery and reduced Wallerian degeneration secondary to this. Restoration of blood supply, particularly proximally from inosculation is essential to satisfactory axonal regeneration [156,259,284,321] as vessel and axon progression mirror each other and are most effective in a well ordered environment. Revascularisation also occurs from the wound bed and the diameter of the graft is known to affect this [3,81,283,322].

In conclusion, sandwich allografts behave in a similar manner to sandwich grafts constructed from other acellular conduits. Generally they improve regeneration from that seen within grafts composed of their parent material here and within the literature. In this study, at an early time point, the results indicate that there may be potential for regeneration to increase with time. Or at least, when regeneration was still evolving for the delay period to be reduced. As hypothesised, the role of the Schwann cell was essential to regeneration. Results indicate that host Schwann

cells repopulated the rejecting nerve allograft and that the additional depot of autologous nerve in the sandwich graft construct proved beneficial in this regard and non-immunosuppressed led to the improvement in regeneration measured within the non-immunosuppressed sandwich allograft compared to its simple equivalent.

Other investigators have concentrated on the benefits this technique confers on achieving greater bridging distances [69,244,245,292,293] . While this remains valid, the potential benefits of reducing the requirements for immunosuppression were of greater interest in this model. Results confirm the hypothesis that within a sandwich allograft without immunosuppression regeneration can be significantly improved. The related reduction in macrophage staining adds further evidence of the reduced antigenicity of this graft type compared to a standard allograft. This suggests that the detrimental effects of immunosuppession withdrawal [151,221,247,266,268] could also be reduced by this technique.

CHAPTER FIVE

Long Term Assessment of Immunosuppressed and Non-immunosuppressed Peripheral Nerve Allografts and Sandwich Grafts

5.1 INTRODUCTION

Previous experiments in Chapters 3 and 4 have shown that without immunosuppression regeneration is poorer in an allograft model although axons which do regenerate show similar regenerative behaviour to that within non-allogenic grafts. The addition of a depot of host Schwann cells to the allograft model as a Sandwich graft improved short term regeneration (see Chapter 4). This introduced the possibility of being able to reduce or abandon immunosuppression use in this allograft model. The depot of Schwann cells appeared to improve regeneration by increasing the efficiency of host Schwann cell repopulation of a rejecting allograft where the donor Schwann cells have been destroyed.

The aim of this experiment was to test whether the short-term advantage conferred by the sandwich allograft model was maintained over a longer time period. Factors affecting long term axonal survival include the satisfactory reinnervation of nerve distal to the level of injury and the re-establishment of appropriate target organ and central connections [4,242,323] . To improve the likelihood of this happening many axonal growth cones are produced from axon sprouts which search for conducive substrates [14,30,31,34,39,52,324] . Once correctly associated with target organs, any unsuitable connections are severed, and the axonal sprouts are pruned [14,52,325,326] . Therefore, the length of time for long-term experiments should be sufficient to allow these processes to take place such that the remaining axons are truly mature and representative.

In the rat sciatic nerve model target organ reinnervation can be established by 12 weeks, and by six months it is more mature although not necessarily fully completed [14,158,216,238,325,327] . However, as allografts have been shown to have slower regeneration potential, these experiments were conducted over 8 months to ensure pruning from all models was essentially complete.

5.2 AIMS

The aim of this investigation was to assess the long-term regeneration potential of non-immunosuppressed nerve allografts to determine whether the addition of a depot of autologous Schwann cells within the construct of a Sandwich allograft could improve regeneration within this allogenic model. Assessments involved morphological analysis of myelinated axons within the posterior tibial nerve distal to the nerve graft, while gastrocnemius muscle mass was measured as an indicator of motor reinnervation and recovery.

5.3 EXPERIMENTAL PROTOCOL

The host and donor animals, along with the operative procedures for graft insertion and specimen retrieval and fixation are detailed in Chapter 2.1. These long-term experiments lasted 32 weeks (Table 5.1). In view of the recognised depot effect which can occur with prolonged subcutaneous administration of CyA [254] , CyA was administered daily up to 12 weeks and on alternate days subsequently. Reports of rejection continuing to a maximum of 9 weeks have been described [328] . In view of this and also that distal end organ reinnervation was predicted to be occurring by 12 weeks (based on the standard regeneration rate of 1mm/day), this time was chosen as the period at which to reduce CyA administration. As in the short-term experiments already described, CyA administration was commenced the evening before surgery.

Oily CyA (oCyA) was used and is made up from base powder CyA donated by Sandoz Pharmaceuticals Ltd., UK. The formulation was prepared and was administered as detailed in Chapter 2.2. The dose used was 5mg/kg/day.

Myelinated nerve fibre analysis was undertaken on thionin blue and acridine orange stained semithin transverse sections of the posterior tibial nerve 5mm distal

to the nerve graft. Analysis was by computerised image analysis as described in Chapter 2.3.4-2.3.5. Gastrocnemius muscle was resected and weighed as in Chapter 2.1.3. Mass was compared between the operated and unoperated muscle and results expressed as a percentage of the normal muscle mass.

Table 5.1 Experimental Groups

Group		Host	Donor	CyA	Numbers
Simple grafts					
Allograft	(Allo+oCyA)	Lew	Da	yes	n=6
Allograft	(Allo-CyA)	Lew	Da	no	n=6
Isograft	(Iso+oCyA)	Lew	Lew	yes	n=5
Isograft	(Iso-CyA)	Lew	Lew	no	n=5
Sandwich grafts					
SandAllo	(SAllo+oCyA)	Lew	Da	yes	n=5
SandAllo	(SAllo-CyA)	Lew	Da	no	n=6
SandControl	(SCon+oCyA)	Lew	Lew	yes	n=6
SandControl	(SCon-CyA)	Lew	Lew	no	n=6
Controls					
Normal		Lew		no	n=6
Unrepaired		Lew		no	n=5
Allograft	(Allo+vehicle)	Lew	Da	no	n=6

5.4 RESULTS

5.4.1 Myelinated fibre analysis

Analysis of semithin sections stained for myelinated axons yielded results from several parameters such as: axon number, size distribution, axon and fibre diameters from which myelin thickness and G-ratio were calculated, along with the measure of laciness. Unequal group numbers evident within the results relate to

technical loss of sections during processing or loss of the animal model within the experimental group (see also Chapter 6).

Table 5.2

Total Axon Counts in Posterior Tibial Nerve – Median values

Simple Grafts	**n**	**CyA**	**Type**	**No. Axons**	**(25-75 centiles)**
Allograft	6	yes	oCyA	1137	(1068-1508)
Allograft	6	no		1235	(931-1583)
Isograft	5	yes	oCyA	1035	(929-1205)
Isograft	5	no		932	(721-1104)
Sandwich Grafts	**n**	**CyA**	**Type**	**No. Axons**	**(25-75 centiles)**
SandAllo	5	yes	oCyA	1125	(801-1222)
SandAllo	6	no		1221	(1069-1760)
SandCon	6	yes	oCyA	926	(755-1138)
SandCon	6	no		1184	(1087-1216)
Controls	**n**	**CyA**	**Type**	**No. Axons**	**(25-75 centiles)**
Normal	6	no		526 *	(474-593)
Unrepaired	5	no		11	(8-15)
Allo+vehicle	6	no		1129	(896-1664)

*$p < 0.05$ Normal vs Allograft+oCyA, Allograft-Cya, SandAllo-CyA, SandControl-CyA.

Comparing the Normal control to all other groups (except Unrepaired), results were not normally distributed. Kruskal-Wallis ANOVA on ranks executed (p=0.001). Dunn's multiple comparison procedure as groups contain unequal numbers.
Results from Unrepaired nerve and Allo+ vehicle were omitted from statistical analysis as gross difference and variability skewed analyses, results included in table for comparison.

The number of regenerated axons were counted as per the description in Chapter 2.3.5 from randomly chosen frames using the image analyser and have been expressed as the median total number of axons (Table 5.2). The frame area was $1.764 \times 10^4 \mu m$. The number of axons counted is greater within the experimental groups than the numbers identified within the normal, unoperated nerve. Statistical analysis identified that this was only significant when comparing the normal group with the non-immunosuppressed sandwich allograft and sandwich control group, plus the simple allograft groups regardless of immunosuppression. All of these groups had counts in the higher ranges for these experiments. The unrepaired group showed the poorest results, which were highly variable. This was a consistent finding when comparing the other parameters under investigation. The nature of the unrepaired group's results affected the statistical analyses especially with regard to their variability, such that this group often skewed the results of analysis. To make more reliable statistical conclusions this group was omitted from formal analysis.

It was surprising to see any evidence of axonal regeneration within the unrepaired group as at specimen retrieval the separated nerve ends were mainly fixed by fibrous tissue onto the muscular bed. Any connection between the neuromas was by fine, tenuous, flimsy connective tissue bands extending between the divided nerve ends across the muscle surfaces. These findings suggest that any neurotisation may have occurred from the muscle rather than the proximal nerve stump (Figure 5.1).

Figure 5.1 Unrepaired nerve at 32 weeks

Distal nerve stump without proximal attachments and tethered to muscular bed.

Results from the control group testing the oil-based vehicle used in the preparation of CyA for injection (Allo+vehicle) also caused problems during statistical analysis. The high variability within this group and also the non-immunosuppressed isograft group produced skewed results. Using the Kruskall-Wallis ANOVA on ranks, statistical analysis produced the same outcome as a One way ANOVA but without the need to omit these latter two groups to improve equal variances.

The results from analysis of frequency distribution of fibre diameters (Table 5.3) show for normal nerve greater amounts of medium and large diameter fibres than the experimental groups. For analysis and presentation, results from the normal group were divided into roughly three equal groups with ranges as shown, allowing easier comparison between the relevant groups.

All experimental groups showed significantly greater amounts of small fibres than normal nerve. The unrepaired group exhibits the highest proportion of small diameter fibres with no contribution within the large diameter range. However, despite this, all experimental groups show no statistical differences.

Table 5.3

Frequency Distribution of Fibre Diameters – Mean Percentage (+/- SD)

Groups	CyA	Type	% Small 1-7μm	% Medium 8-10μm	% Large 11-20+μm
Simple Grafts					
Allograft	yes	oCyA	87.77 (+/-2.73)	9.42 (+/-2.04)	2.80 (+/-0.84)
Allograft	no		90.76 (+/-5.66)	7.63 (+/-4.22)	1.61 (+/-1.47)
Isograft	yes	oCyA	87.00 (+/-7.08)	9.98 (+/-4.65)	3.03 (+/-2.81)
Isograft	no		90.13 (+/-3.73)	8.15 (+/-2.90)	1.72 (+/-0.85)
Sandwich Grafts					
SandAllo	yes	oCyA	86.51 (+/-5.03)	10.23 (+/-3.15)	3.27 (+/-2.14)
SandAllo	no		87.48 (+/-4.47)	9.55 (+/-2.91)	2.97 (+/-1.63)
SandCon	yes	oCyA	87.87 (+/-2.87)	9.24 (+/-1.97)	2.89 (+/-1.49)
SandCon	no		88.32 (+/-4.95)	9.26 (+/-2.98)	2.42 (+/-2.09)
Controls					
Normal	no		33.39 * (+/-4.35)	31.56 * (+/-5.12)	35.05 ** (+/-9.33)
Unrepaired	no		98.00 ** (+/-4.47)	2.00 (+/-4.47)	0.00 (+/-0.00)
Allo+vehicle	no		90.30 (+/-5.17)	8.06 (+/-3.85)	1.64 (+/-1.36)

Comparing % **Small** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.
Comparing % **Medium** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.
Comparing % **Large** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, **$p<0.001$ Normal vs All groups.

Omitting the Normal group. One way ANOVA, $p=0.049$ showing no significant difference between the remaining groups.

For the medium and large fibre diameter ranges, the normal nerve group shows greater distribution here than all the other groups, which is significant. Repeating the analysis without the normal group showed no statistical difference between the experimental groups within either of these ranges. The results for groups receiving CyA show better, although non-significant, fibre distribution within the mid-high diameter ranges, as does the sandwich allograft without immunosuppression. These results suggest that both immunosuppression and the sandwich graft are beneficial.

Tables 5.4 – 5.6 show the results from measurement of axon and fibre diameter, myelin thickness, G-ratio and laciness. These measurements represent the mean results from 3 random frames of analysis from at least two section per animal, from which the group's descriptive statistics were calculated.

Table 5.4 Myelinated Nerve Morphology – Mean +/- SD

Group	n	CyA	Type	Axon Diameter µm	Fibre Diameter µm
Simple Grafts					
Allograft	6	yes	oCyA	1.78 (+/-0.14)	4.47 (+/-0.25)
Allograft	6	no		1.76 (+/-0.37)	4.21 (+/-0.44)
Isograft	5	yes	oCyA	1.99 (+/-0.39)	4.55 (+/-0.54)
Isograft	5	no		1.69 (+/-0.26)	4.29 (+/-0.37)
Sandwich Grafts					
SandAllo	5	yes	oCyA	1.97 (+/-0.26)	4.42 (+/-0.32)
SandAllo	6	no		1.75 (+/-0.20)	4.42 (+/-0.29)
SandCon	6	yes	oCyA	1.72 (+/-0.26)	4.28 (+/-0.26)
SandCon	6	no		1.63 (+/-0.20)	4.38 (+/-0.38)
Controls					
Normal	6	no		4.70 (+/-0.48) *	8.39 (+/-0.62) *
Unrepaired	5	no		1.24 (+/-0.27)	3.13 (+/-0.34) **
Allo+vehicle	6	no		1.87 (+/-0.35)	4.29 (+/-0.51)

Comparing **Axon diameters** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.

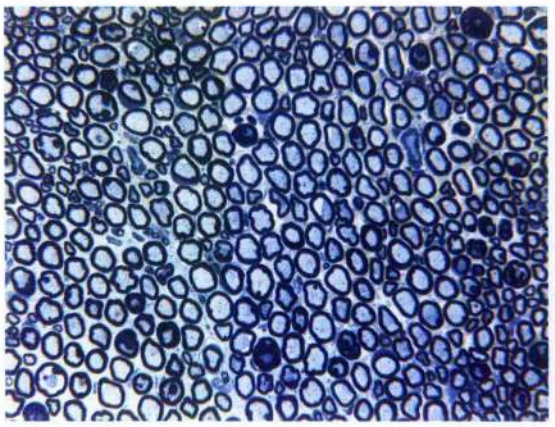
Comparing **Fibre diameters** One Way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups; **$p<0.04$ Unrepaired vs Allograft+oCyA, Isograft+oCyA, SandAllo-CyA.

The measurement of fibre diameter reflects the degree of fibre myelination (Table 5.4). The normal group's results were significantly better than all other groups. The unrepaired group, although poorest, is only significantly different from the groups with some of the highest values namely the immunosuppressed simple allograft and isograft, and the sandwich allograft without immunosuppression. Results comparing the unrepaired group with the immunosuppressed sandwich allograft and the non-immunosuppressed sandwich control group were equivocal with p values of 0.059 and 0.052 respectively. The interpretation of measurements from the unrepaired group tends to indicate superior regeneration to that indicated by the appearance of the nerve sections from this group and the raw data (Figure 5.2). This highlights the fact that although statistics provides a valuable tool for describing and comparing data, it is only one factor while the overall picture still has to be appreciated in the interpretation.

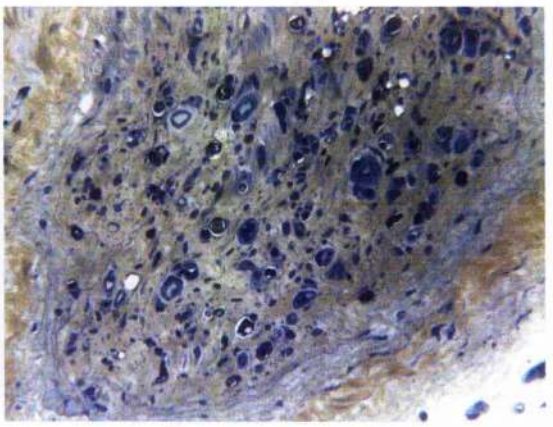
Regarding axon diameter (Table 5.4), the normal nerve shows significantly the largest mean value, while the unrepaired group shows the lowest value. However, none of the experimental groups' results show any significant differences for axon or fibre diameters.

Figure 5.2 Semithin sections of distal myelinated nerve x 40

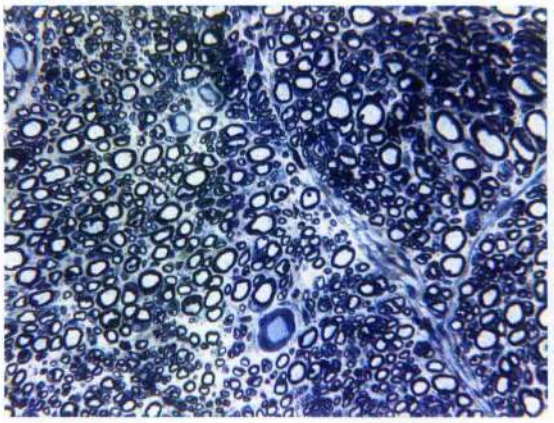
Normal nerve



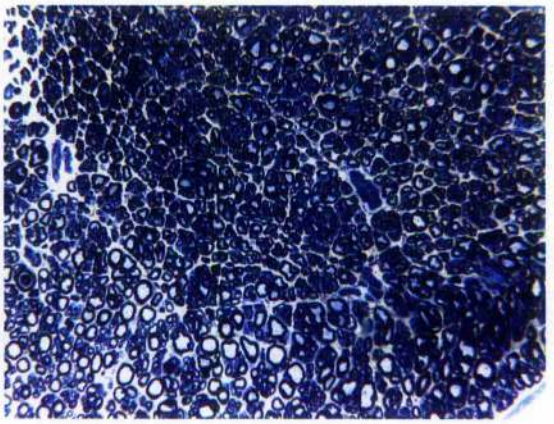
Unrepaired distal nerve stump



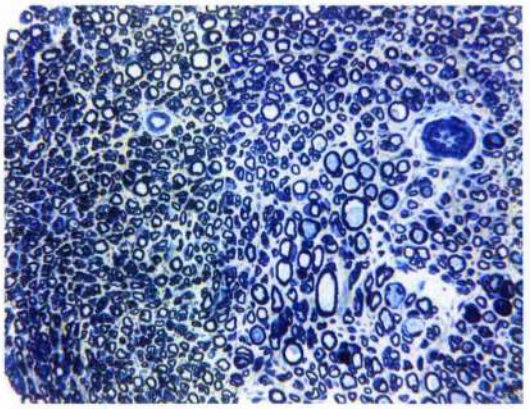
Allo+oCyA



Allo-CyA



SandAllo+oCyA



SandAllo-CyA

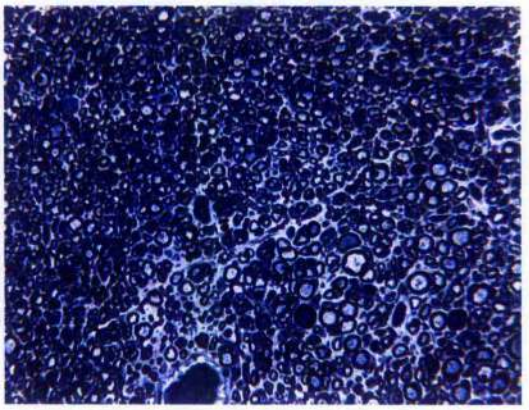


Table 5.5 Myelinated Nerve Morphology – Mean +/- SD

Group	n	CyA	Type	Myelin Thickness µm	G-ratio
Simple Grafts					
Allograft	6	yes	oCyA	1.35 (+/-0.10)	0.34 (+/-0.03)
Allograft	6	no		1.22 (+/-0.08)	0.36 (+/-0.04)
Isograft	5	yes	oCyA	1.28 (+/-0.12)	0.38 (+/-0.04)
Isograft	5	no		1.30 (+/-0.06)	0.34 (+/-0.03)
Sandwich Grafts					
SandAllo	5	yes	oCyA	1.23 (+/-0.06)	0.37 (+/-0.02)
SandAllo	6	no		1.34 (+/-0.09)	0.33 (+/-0.03)
SandCon	6	yes	oCyA	1.28 (+/-0.05)	0.34 (+/-0.03)
SandCon	6	no		1.38 (+/-0.12)	0.32 (+/-0.03)
Controls					
Normal	6	no		1.85 (+/-0.09) *	0.50 (+/-0.01) **
Unrepaired	5	no		0.58 (+/-0.48)	0.26 (+/-0.22)
Allo+vehicle	6	no		1.21 (+/-0.14)	0.39 (+/-0.05)

Comparing **Myelin Thickness** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.

Comparing **G-ratio** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, **$p<0.002$ Normal vs all groups. Unrepaired group omitted from statistical analysis in view of gross difference and high variability, results included in table to indicate this.

Thinner myelination of fibres would be expected in the experimental groups compared to the normal nerve, and analysis of the results confirms this (Table 5.5), with the normal nerve exhibiting statistically greater myelination than all the operated groups. The unrepaired group has obviously inferior results than the others and was omitted form statistical analyses for reasons expressed previously.

The G-ratio is calculated as the proportion of the axon diameter to the myelinated fibre diameter, and therefore it reflects the degree of myelination. There is some disagreement within the literature as to the relationship between myelin thickness and fibre diameters between normal and regenerated nerves. Some report a parabolic relationship where the smallest fibres exhibit low (<0.4) G-ratios [329], while others show a linear relationship, or no appreciable relationship at all [330,331]. The normal group results here are statistically superior to the other groups. The

lower G-ratios associated with the regenerated axons in the experimental groups support the parabolic argument where the increased proportion of smaller fibres within the nerve skews the mean G-ratio. This is because very small fibres often have proportionally thicker myelin in an attempt to restore their conductive properties [52] . In this format (Table 5.5), the exact relationship between G-ratio and fibre dimensions are obscure, but it serves to identify the difference between normal and regenerated nerves.

Table 5.6 Myelinated Nerve Morphology – Mean +/- SD

Group	n	CyA	Type	Laciness	SD
Simple Grafts					
Allograft	6	yes	oCyA	0.82	(+/-0.01)
Allograft	6	no		0.84	(+/-0.03)
Isograft	5	yes	oCyA	0.84	(+/-0.02)
Isograft	5	no		0.82	(+/-0.03)
Sandwich Grafts					
SandAllo	5	yes	oCyA	0.84	(+/-0.03)
SandAllo	6	no		0.79	(+/-0.01)
SandCon	6	yes	oCyA	0.83	(+/-0.02)
SandCon	6	no		0.83	(+/-0.03)
Controls					
Normal	6	no		0.85	(+/-0.02)
Unrepaired	5	no		0.51	(+/-0.42)
Allo+vehicle	6	no		0.80	(+/-0.03)

One way ANOVA no significant difference between experimental groups

Unrepaired group omitted from statistical analysis in view of gross difference and high variability, results included in table to indicate this.

Laciness relates to the shape of the fibres (Table 5.6). A value of 1 indicates a perfect circle with smaller values indicating more irregular shapes due to fibre damage or imperfect regeneration. The unrepaired group gives the poorest value but was omitted from analysis because of its high variability. Otherwise, most groups including the normal group show no significant difference.

5.4.2 Target Reinnervation

For target organ reinnervation, motor reinnervation of gastrocnemius muscle was assessed by comparing the mass of the muscle on the operated side with that of the normal muscle. Each animal therefore contributed its own control (Table 5.7).

Table 5.7 Gastrocnemius muscle mass – % operated side / normal

Simple Grafts	n	CyA	Type	Median %	25-75 centiles
Allograft	6	yes	oCyA	64.24	(53.98-70.90)
Allograft	6	no		68.21	(59.05-72.93)
Isograft	5	yes	oCyA	58.91	(52.27-63.52)
Isograft	5	no		49.29	(35.62-59.40)
Sandwich Grafts					
SandAllo	5	yes	oCyA	65.00	(61.37-65.67)
SandAllo	6	no		72.39	(69.91-74.69)
SandCon	6	yes	oCyA	68.20	(60.23-75.42)
SandCon	6	no		67.75	(65.63-68.61)
Controls					
Unrepaired	5	no		11.68 */**	(9.69-13.11)
Allo+vehicle	6	no		66.56	(51.83-74.68)

Results not normally distributed therefore Kruskal-Wallis One way ANOVA on ranks executed using Median values.

Comparing Unrepaired to Simple grafts (including Allograft+vehicle), K-W ANOVA, p=0.002. Dunnett's multiple comparison procedure, *p<0.05 Unrepaired vs All groups.

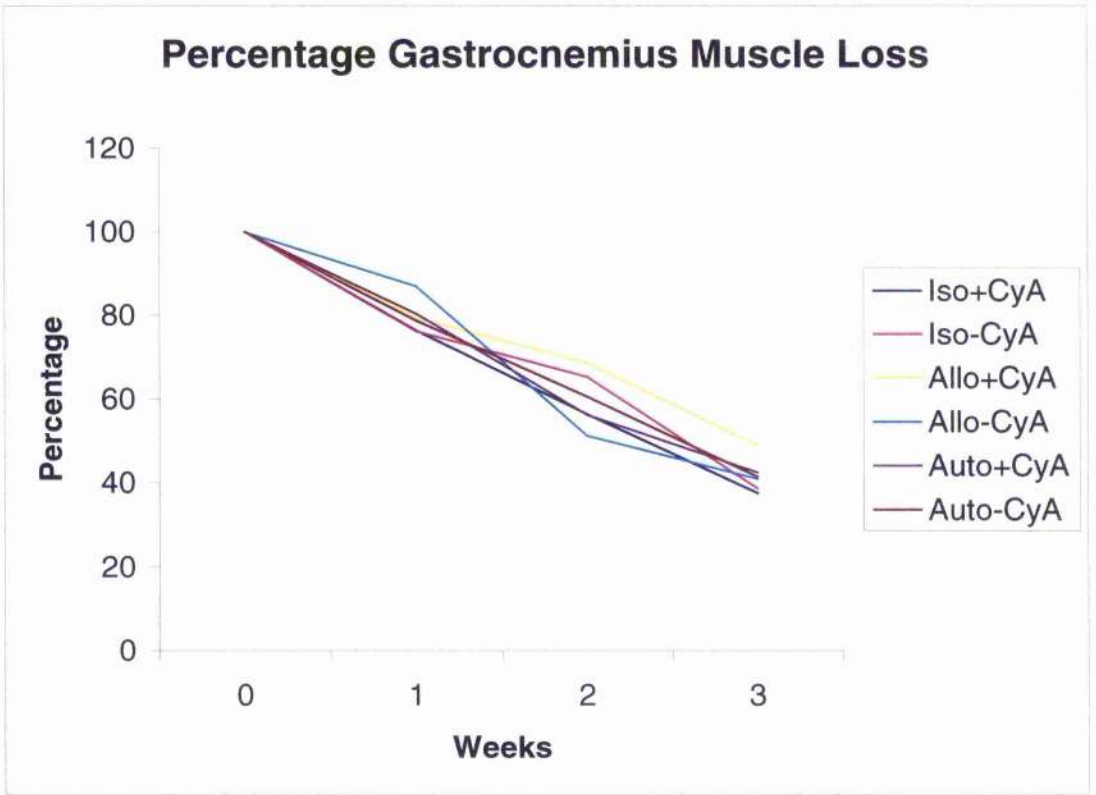
Comparing Unrepaired to Sandwich grafts, K-W ANOVA, p=0.001. Dunnett's multiple comparison procedure, **p<0.05 Unrepaired vs All groups.

Omitting Unrepaired group, K-W ANOVA shows no significant difference between the remaining groups, p=0.061.

The unrepaired group shows significant muscle atrophy and highly variable results as experienced within the other assessment modes. All experimental groups had significantly greater muscle mass than the unrepaired group being around two-thirds the mass of normal muscle. There was no significant difference noted comparing the experimental groups although the best muscle weight was recorded in the non-immunosuppressed sandwich allograft group, while the standard isograft control groups had the lowest results.

Analysis of mass of Gastrocnemius muscle harvested from experimental groups within the early regeneration period (Chapter 3), show a steady reduction in muscle mass following denervation from 7-21 days (Figure 5.3). There were no significant differences found between groups at any time point.

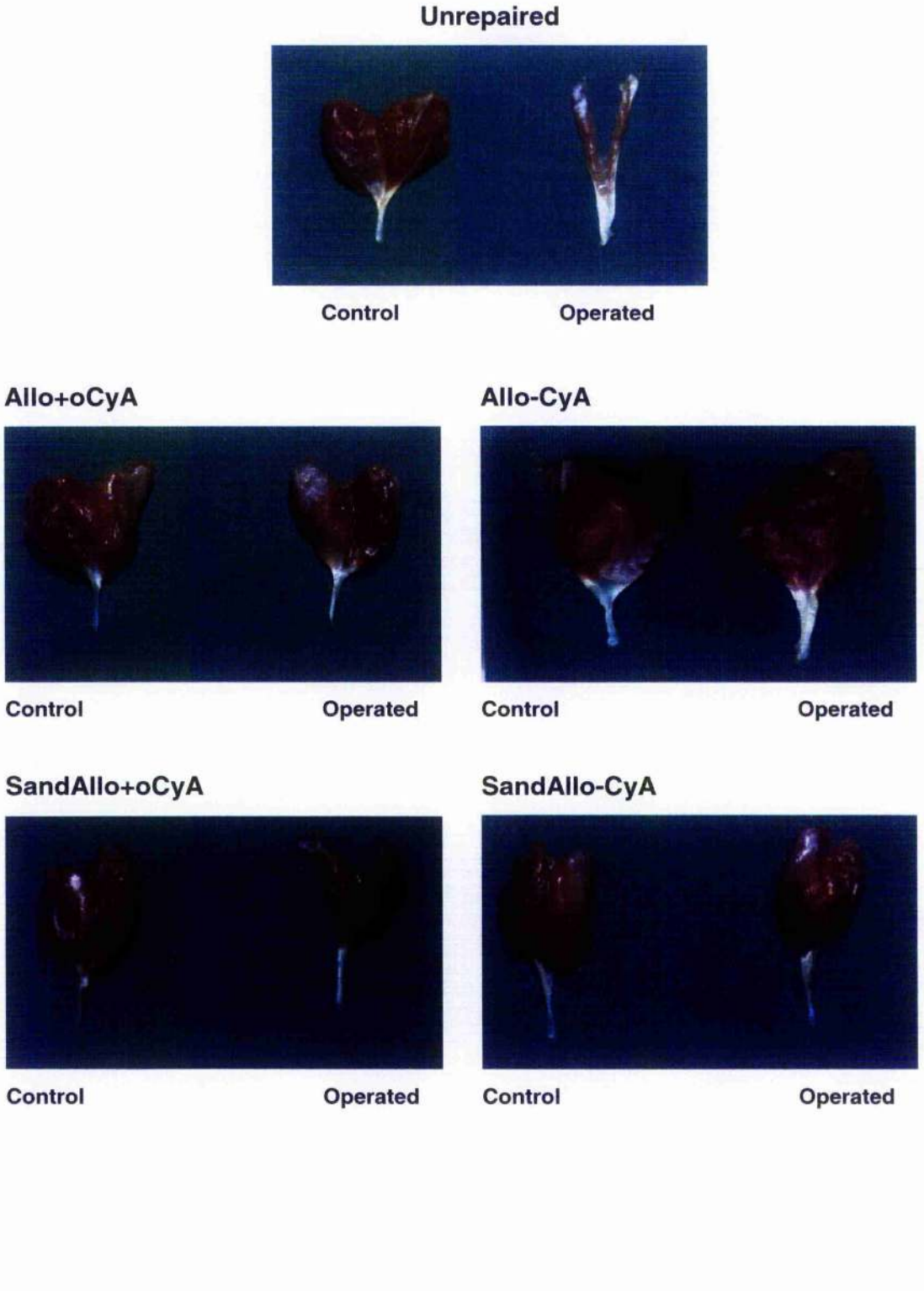
Figure 5.3 Gastrocnemius muscle mass following denervation



The rat, despite having a major nerve trunk injury, continues to take weight on its affected hind limb throughout the experimental period. The limb also contributes to ambulation although the gait is altered through changes to ankle, knee and foot contribution. Therefore, although the uninjured limb will undertake compensatory work following the injury, all of the rats were similarly affected. The muscle was undisturbed by the initial surgery and on removal all muscles appeared normal and healthy, with no evidence of abnormal fibrotic or adipose tissue which could have contributed to their mass (Figure 5.4).

Overall, these results indicate that regardless of immunosuppression, in this animal model, simple allografts and sandwich allografts exhibit similar regeneration profiles to the isograft groups which represent current clinical practice. The use of a sandwich graft did not confer any statistically significant benefit, or deficit, although across some parameters it did exhibit results closer to normal values than other experimental groups.

Figure 5.4 Gastrocnemius muscle at 32 weeks



5.5 DISCUSSION

Following nerve injury and repair we have previously shown that axonal regeneration is established in non-allogenic nerve grafts by three weeks. Regenerating axons have to regenerate to reach target organs and form appropriate connections. It is understood that axons regenerate at an average of 1mm per day [14,41] . However this rate varies from 1-8.5mm/day, being inversely proportional to the distance the injury is from the neurone [3,36,41] . Regeneration sufficient to reestablish target organ connections within 12 weeks has been noted in the rat. The mechanism whereby axons regenerate leads to the production of more axon sprouts than required [14,30,31,34,52,324] . This process improves the likelihood of an axon finding a suitable substrate for regeneration, thereby reestablishing appropriate innervation with Schwann cells en route and finally connecting the correct neurone with its target organ [14,34,38,39] . Once the primary neuronal unit has been reestablished, any superfluous axons with inappropriate connections die back through the process of pruning. Pruning can occur at any point and can last for some time. However, as serial tests of function up to a year following sciatic nerve repair have shown a plateau is reached around 12 weeks, the peak of pruning is likely to be largely complete by around this point [216,325,327] . As our previous work has identified that regeneration in an allogenic model is delayed by comparison with non-allogenic models the longer time period of 8 months was chosen to ensure mature axonal regeneration in these experiments.

The final outcome of nerve repair depends on the correct connection between the central nervous system, peripheral neurone and target organ where signals can pass in an antegrade and retrograde manner [4,242,323] . Functional assessments are notorious for their subjectivity, qualitative nature and difficulties with interpretation [192,332,333] . Indeed there are no "routine" functional tests experimentally or clinically where there is uniform agreement regarding the relative merits of one test over another.

In this experiment, analysis of myelinated nerves present within the posterior tibial nerve distal to the experimental nerve graft was undertaken. This morphological technique gives quantitative information related to the maturity and success of nerve regeneration within the distal nerve. Although not testing function, measurements of axonal size and myelin thickness are known to correlate with electrophysiological functional assessments [2,210,219], this is because of the relationship between axon diameter, myelin thickness and the internode distance [52,331].

Muscle atrophies when denervated, which is reversible in a time dependant manner if innervation is reestablished. Hence the return of muscle bulk indicates the return of innervation and with this, functional potential [334]. However, prolonged denervation atrophy is associated with connective tissue fibrosis, impeding the establishment of end-plate formation and nerve regrowth [3,89]. Sterne used gastrocnemius muscle mass as an end-point indicator of reinnervation after using fibronectin conduits impregnated with the neurotrophic factor, Neurotrophin 3 (NT-3) in comparison to autograft controls [65]. Initial muscle atrophy was followed for 30 days post engraftment showing that the results here at 21 days do not represent the lowest mass reached. At 34 weeks autografts had attained 71% of their control muscle mass [65], producing similar results to those shown here. Other authors have also found recovery of different muscle mass to reach approximately 70% of control muscle mass following nerve repair [209,334,335].

From the results obtained from these experiments it can be seen both functionally and morphologically that the results from grafted and regenerated nerves fall short of normality. However, the similar values across the experimental groups are indicators that regeneration has reached a stable phase, especially in relation to the isograft groups, and the morphological features as shown in the figures (Figure 5.2).

Statistical analysis has shown that some of the groups are similar to the normal nerve in terms of myelinated axon numbers, although the absolute axon number is not always the most useful parameter. The experimental groups have a higher number of axons than the normal nerve as nerve pruning may still be taking place [14,52,325,326] . Therefore other variables must be considered. As there is no significant difference between measures of axon, fibre diameter, myelin thickness or when considering the qualitative features of the experimental groups or muscle mass, then the lower values values for axon counts in the isograft groups, the immunosuppressed sandwich allograft and sandwich control groups appear to suggest the lower numbers indicate more appropriate axonal connections.

It is recognized that axons regenerating post injury are smaller and with thinner myelin than that noted in normal nerve [52,330,336] . The alteration in these parameters is evident from the results shown here. As conduction velocity correlates to axon and myelin dimensions [2,210,219] , then the reduced function related to these dimensions can be appreciated without electophysiological testing. The recovery of muscle mass indicates the reinervation of muscle fibres but not up to normal levels, although significantly better than in the unrepaired group.

Comparing the distribution of fibre diameters, there were no statistical differences between experimental groups, although there were fewer numbers of medium and large fibres within the non-immunosuppressed allografts group which suggests a possible benefit being conferred by CyA and the host section within the sandwich allograft.

Following nerve injury, the restoration of normal function is the exception rather than the rule [11,36,41,48,289,337] . For this reason, autografting, as the usual method of nerve reconstruction is the standard by which all other techniques are compared. The IsoG-CyA group represents the standard non-allogenic graft technique in

these experiments. For all of the assessments here there are no significant differences determined between experimental groups. The use of the sandwich grafting technique, where a graft of host nerve is transplanted orthotopically within an allogenic graft model forming a chimeric structure, is validated by these experiments.

The use of a sandwich control group was to determine whether the extra neurorrhaphies were deleterious. Using autologous muscle grafts Whitworth showed that over short (2.2cm), and long (5cm) graft distances the effect of the sandwich graft was beneficial [245,315] . However, Maeda found a 5 segment silicone /nerve sandwich model (24mm) did appear to hinder regeneration, but not an 18mm model [244] . In the sandwich graft model the addition of a depot of cellular nerve can outweigh the potential deficit of an extra neurorrhaphy where the host Schwann cells have repopulated acellular muscle [245,315] , silicone conduits [244] , vein [69,293,311] and now rejected nerve allograft to aid axonal regeneration.

These experiments show that in a non-immunosuppressed allogenic model, where donor Schwann cells are reduced (see Chapter 3.4.6-3.4.7), the addition of a host nerve segment containing its complement of Schwann cells aids regeneration of host axons through the graft construct. Results in Chapter 3 and 4 show that over short gaps of 1 and 1.5cm respectively, axonal regeneration does occur within the simple non-immunosupressed allograft. However the extent of regeneration within the early time points is much reduced compared to non-allogenic groups. As predicted from other work [78,151,221,247,263,264,268] and the evidence of reduced Schwann cell staining here, rejection is occurring, but regeneration progresses in a similar fashion to the non-allogenic models as shown by examination of longitudinal histological sections.

In our experiments we have shown that a sandwich graft technique conferred a benefit on regeneration through a non-immunosuppressed allograft up to 21 days. It was also evident that the benefits continued to be observed up to 32 weeks, as no difference was seen between groups regardless of immunosuppression. These results are at odds with other investigators. The majority of opinion would indicate that nerve allografts are poorer than auto or isografts unless immunosuppressed [78,97,124,153,204,209,268]. With immunosuppression, results from allografts however are comparable to non-allogenic grafts [153,206,211,216,223,247]. In the experiments described here no such differences were noticed long term. The reasons for the differences may be that the model is too efficient at regenerating, that the gap distance is too small, that the immunosuppression was inadequate, or that insufficient genetic disparity existed between the rat strains.

The rat is a commonly used experimental model. The strains used here have been used before in transplantation experiments [151,162,220,230,247,338] and are known to have a mismatch at both major and minor histocompatability loci [220,246,248]. In our previous experiments on simple allografts (see Chapter 3.4) we have shown by qualitative and quantitative morphological assessment of Schwann cell and macrophage staining that immunological rejection had occurred. The use of CyA at the recommended dose (5mg/kg/day) [206,254,262] produced improved axonal regeneration, along with reduced macrophaged infiltration and greater Schwann cell numbers. Hence, there would appear to be sufficient genetic disparity between the rat strains.

Nerve regeneration in the rat is generally faster than in higher mammals [14,339], and one could argue that the use of a short gap of less than 1.5cm in a rat is insufficient to hinder regeneration [52,55,192,211,250,340]. Such short gaps can allegedly be bridged successfully without grafting or repair. However, we have shown that regeneration in unrepaired controls was not successful. Such innervation as was observed was minimal and very variable, consequently many statistical comparisons between

groups were impossible. All myelinated axons were of small diameter, and generally the fibres showed poorer evidence of regeneration across all parameters. Morphologically the nerve sections were smaller and more fibrotic than the other groups. At retrieval, any neurotisation which had occurred did not appear to emanate from the proximal nerve stumps.

Results from Chapters 3 and 4 along with evidence of work using acellular conduits of organic, inorganic or synthetic material indicate that host Schwann cells will repopulate the acellular structures and improve regeneration [62,69,75,76,238,239,244,245,290-293] . The addition of host Schwann cells within the sandwich allograft had obvious early benefits. While these remained evident in the non-immunosuppressed sandwich group at the 32 weeks assessment the benefit over the simple non-immunosuppressed allograft group was obscure. Even this group at the end of the experiment had regenerated similarly to the other experimental groups. This may relate to the size of the nerve gap. It is recognised that the longer the bridging conduit the poorer the regeneration [52,68,319] . This has been attributed to the reduced gradient of positive regeneration influences the further the regenerating axon front is from the distal nerve [40,52,55,68] . While nerve tissue [69,245,293] , Schwann cells [61,62,77,162,238,240] , or addition of neurotrophic factors [4,52,65,241-243] will improve regeneration distances, as yet the distance regeneration has to proceed along remains a major factor in determining outcome. Proximal injuries fair worse than distal for this reason, but also because there are more nerve branches within proximal nerve trunks which have to be negotiated by the regenerating axons on their route distally [3,45,341] . This provides many opportunities for incorrect innervation which despite the arrangement of the growth cone, neurotrophic, neurotropic and contact guidance cues does not ensure ideal regeneration.

We have already indicated that the dose of CyA administered was associated with the prevention of the negative effects of allografting shown in Chapters 3 and 4

such as increased macrophage infiltration and reduced Schwann cell staining. The difference between the formulations used was that for the long term experiments a less expensive but still frequently used oily preparation was employed and recommended by the manufacturer (Sandoz) [210,213,251,253,254] . The depot effect of oily CyA and risk of infection in injection sites are known from the work of Bain and Midha [206,254,262] and for these reasons the injection site was varied as recommended and the dose of CyA was reduced by alternating the days of injection. To minimise any effect on regeneration we waited until target organ reinnervation was likely to be underway at 12 weeks before altering the CyA regime. The addition of a control group using the vehicle used to prepare the CyA for injection was to verify that no additional benefit was incurred by administration of this solution. The results for the AlloG+vehicle group are similar to the Allo-CyA group and all the other groups for the reasons given previously.

Investigations of temporary immunosuppression for peripheral nerve allografting have produced some controversial results. However, the differences in outcome can often be related to study design, such as the absence of a distal coaptation [123,125,195,204] , insufficient immunosuppression to allow target reinnervation [153,168] , and lack of appropriate controls [210,211,216] . MacKinnon and collaborators are probably the strongest advocates of temporary immunosuppression and although they noted an axonopathic process morphologically, electrophysiologically and functionally, this was reversible with time [134,207,216,218] . They have investigated peripheral nerve allografting in primates [129,133] and have gone on to undertake it in humans. MacKinnon has reported 7 clinical cases of nerve allotransplantation using CyA, Fk506, azathioprine and prednisolone between 1988 and 1998 [217,224,225] . She has combined allo- and autografting but as parallel unconnected cables rather than in series, as here. This was explained as a fail-safe strategy if the nerve allograft failed, and also to decrease the antigenic load of the combined cable graft structure [224] .

As noted in Chapter 3 there did appear to be a small benefit conferred by CyA administration to non-allogenic and allograft groups alike. This difference was not significant. Work with CyA [134,228,288] and FK506 [226,227,342,343] have shown that immunosupressant administration can improve regeneration in allografts and autografts. FK506 is a more potent immunosupressant such that more beneficial regeneration in allografts has been noted with it than with CyA [226,227,344] . As parenteral CyA was used in the experiments in Chapters 3 and 4 and in view of the similarity of results among the experimental groups here we have performed the same experiments again using parenteral CyA and have repeated the early experiments using oily CyA. These results are reported in Chapter 6.

CHAPTER SIX

Assessment of the effects of Cyclosporin preparations on Axonal Regeneration and on Well-being of Nerve Graft Recipients

6.1 INTRODUCTION

6.2 AIMS

6.3 EXPERIMENTAL PROTOCOL

6.4 RESULTS

6.4.1 Early regeneration – 21 days

6.4.1.1 Axonal regeneration

6.4.1.2 Macrophage quantification

6.4.1.3 Schwann cell quantification

6.4.2 Long-term regeneration - 32 weeks

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6.4.2.2 Gastrocnemius muscle mass

6.4.3 Cyclosporin monitoring

6.4.3.1 Cyclosporin levels

6.4.3.2 Hepatic and renal function

6.4.3.3 Weight gain

6.4.4 Morbidity and Mortality

6.5 DISCUSSION

6.1 INTRODUCTION

Since the establishment of allotransplantation and the development of Cyclosporin (CyA) and the newer generation immunosuppressants, researchers have concentrated efforts towards testing hypotheses and techniques which may reduce immunosuppressant requirements [105,154,168,192,301,302,333,345-350] . Unlike in clinical practice, CyA was shown to be a useful single agent immunosuppressant for nerve allografting in rats [97] . This has made comparison and reproducibility of experiments much simpler. Experience with CyA and knowledge of its toxicity has made the establishment of safe dosage regimes essential. Several factors are known to interplay in relating drug dose to action. These include: route of administration, bioavailability, phamacokinetics and pharmacodynamics of the agent [202,213,256] . Compliance is an important issue in human practice, with ease of administration to ensure correct dose delivery being a similar factor in veterinary practice.

CyA is not water soluble [198,200,202] . Sandoz have developed a parenteral formulation which can be diluted in physiological fluids and have also altered their oral preparation to improve its bioavailability [214,255,351] . However, for nerve allograft research many investigators have used base powder CyA from Sandoz, which needs to be dissolved in an oil and alcohol mixture (see Chapter 2) [202,206,213,253,262] . From experience of preparing solutions of CyA, it would be expected that parenteral CyA would have greater bioavailability than oily CyA.

Some of the issues involved in the pharmacokinetics of CyA administration in the rat have been already examined, especially regarding an optimal route of administration [213,262] . Comparing oral, intramuscular, intraperitoneal and subcutaneous routes of administration of an oil based preparation, Wasseff found that although the subcutaneous route did not produce the greatest bioavailability, it did achieve the most stable serum concentration of CyA. Oral administration was

poorest [213] . Following on from this, Bain determined that a dose of 5mg/kg/day was the lowest effective dose in providing immunosuppression sufficient for peripheral nerve allografting in rats, thus reducing the risks of CyA toxicity [206] . Histological features of an inlaid nerve allograft and the response to a mixed lymphocyte reaction immunological test were used to compare allogenic and non-allogenic groups. Both of these authors also noted that increasing harmful effects were associated with higher CyA doses and concentrations.

For short-term experiments using low doses, toxic effects were not a great problem, however as nerve regeneration takes time Midha expressed some concern over long-term CyA use in rats. His study identified a depot effect of using oral-type formulations of CyA subcutaneously (oil based CyA preparations) which resulted in greater toxicity from CyA [254] . The best indicator of toxicity was the animals' weight gain which fell drastically following CyA administration and although recovering with time, failed to rise in line with normal rats' weight. Farthings earlier work also defined weight as a good indicator of CyA toxicity [253] . He also noted that failure to thrive and changes to liver and kidney (functionally and histologically) were related to dose and duration of CyA administration.

All of the above contributions have stressed the importance of achieving a safe therapeutic window for CyA use. In this project the dose of 5mg/kg/day was used and to prevent an excessive depot effect, the CyA was administered on alternate days after 12 weeks, which was in line with Midha's work.

In Chapter 5, essentially no major differences were noted between the experimental groups regardless of immunosuppression or graft type. The reasons for this could relate to the use of oily CyA and to lack of sufficient genetic disparity between the rat strains. As Chapter 3 results did indicate poorer regeneration within the allografted nerve with loss of donor Schwann cells and an increased

macrophage response, most likely as part of a rejection response, then there would appear to be sufficient evidence to discount genetic similarity between the strains [151,162,246] . However, further investigation is required to test whether the CyA preparations are truly adequate.

6.2 AIMS

To test the hypothesis that oily CyA(oCyA) may not provide sufficient immunosuppression for nerve allografts and sandwich grafts, oCyA had to be assessed compared to parenteral CyA (pCyA), which had been shown to be successful over the early regeneration period. Axonal regeneration, Schwann cell and macrophage staining were compared up to 21 days (see Chapters 3 & 4). Long-term results from pCyA groups were compared to results from oCyA (see Chapter 5) to complete the analysis.

As oCyA is hypothesised to be less bioavailable and more associated with depot effects than pCyA, this could be expected to affect CyA concentrations and therefore toxicity. A further aim of this project was therefore to determine any potential harmful effects which may have resulted from these experiments by measuring animal weights, serum CyA concentrations, hepatic transaminases, alkaline phosphatase and creatinine as indicators of hepatic and renal function respectively.

6.3 EXPERIMENTAL PROTOCOL

Within the early regeneration period of 21 days, simple graft procedures were employed as in Chapter 3. Ten millimetre grafts were transplanted orthotopically following resection of 10mm right sciatic nerve segments.

For 32 week long-term experiments the protocol was similar to that in Chapter 5. Ten millimetre sciatic nerve resections were repaired with 15mm grafts. In this

situation autografting without sacrifice of an additional nerve is impossible, therefore isografts formed the main non-allogenic control group (Table 6.1).

Cyclosporin remained the sole immunosuppressant used. Results have already been reported on the parenteral preparation of CyA (pCyA) in Chapters 3 and 4, and on oily preparation of base powder CyA (oCyA) in Chapter 5. Therefore to complete the assessment of both CyA preparations in this experiment groups receiving oCyA and pCyA were undertaken and assessed over 21 days and 32 weeks respectively.

Assessments involved analysis of axonal, Schwann cell and macrophage staining at 21 days, and myelinated fibre characteristics at 32 weeks. In addition comparisons were made of gastrocnemius muscle mass.

To assess the general effects of CyA administration, as well as daily observations, the animals receiving CyA were weighed weekly. They also had blood samples taken for analysis of CyA levels, serum creatinine (creat), alkaline phosphatase (alk phos), alanine-amino transferase (ALT) and aspartate-amino transferase (AST) as indicators of renal and hepatic function. Non-immunosuppressed animals were weighed at operation and at the end of the experiments.

Table 6.1 Experimental Groups

Group	Code	Time (weeks)		CyA	Numbers
Simple grafts					
Allograft	(Allo+oCyA)	3	32	oily	6
Allograft	(Allo+pCyA)	3	32	parenteral	6
Allograft	(Allo-CyA)	3	32	none	6
Isograft	(Iso+pCyA)	3		parenteral	5
Isograft	(Iso+oCyA)		32	oily	5
Isograft	(Iso-CyA)	3	32	none	6
Sandwich grafts					
Sand.Allo	(SAllo+oCyA)		32	oily	6
Sand.Allo	(SAllo+pCyA)	3	32	parenteral	6
Sand.Allo	(SAllo-CyA)	3	32	none	6
SandControl	(SCon-CyA)	3	32	none	6

6.4 RESULTS

6.4.1 Early Regeneration – 21 days

6.4.1.1 Axonal regeneration

Macroscopically, the harvested allograft immunosuppressed with oily CyA (Allo+oCyA) had a yellower colour than the corresponding allograft segment from the group immunosuppressed with parenteral CyA (Allo+pCyA). However, the colour was not as obvious, nor the grafts as firm as the non-immunosupressed allografts (Allo-CyA).

Table 6.2

21 day Percentage area of axonal staining – Proximal Allograft Mean (+/-SD), n=6

	+pCya	+oCya	-CyA
Allograft	19.66*	14.13	11.89
	(+/-1.79)	(+/-4.14)	(+/-4.64)

* $p < 0.05$ Allograft+pCyA vs Allograft-CyA

One way ANOVA to compare groups (p=0.008). Tukey multiple comparison procedure to compare every pair of group means.

Quantification of axonal regeneration within the proximal graft (Area G – Figure 2.4) indicated similar findings to the macroscopic features. The Allo+oCyA group produced axonal regeneration values between those of the Allo-CyA and Allo+pCyA groups (Table 6.2). Comparisons were also made with the allogenic and non-allogenic grafts from Chapter 3 (Table 6.3). These show that the Allo-CyA and Allo+oCyA groups were significantly different from the Isograft control groups, while the Allo+pCya group was not.

Comparisons were also made with the sandwich graft groups from Chapter 4 (Table 6.4). Although the sandwich grafts in these experiments are 5mm longer than the simple grafts, the analyses were all undertaken within the same proximal quantification zone (Area G) to allow comparative assessments. Within this comparison, the Allo+oCyA group and the Allo-CyA group showed consistently statistically poorer axonal regeneration than the Allo+pCyA group. However the Allo+oCyA group showed similar results to those of the sandwich graft groups, who in turn produce similar results to those from the Allo+pCyAgroup.

Table 6.3

21 day Percentage area of axonal staining – Proximal Simple Graft Mean (+/-SD), n=6

	Allograft	Autograft	Isograft
+pCyA	19.66 (+/-1.79)	18.37• (+/-2.78)	25.88 (+/-3.24)
+oCyA	14.13** (+/-4.14)		
-CyA	11.89* (+/-4.66)	28.84•• (+/-2.68)	24.07 (+/-7.04)

* p<0.05	Allograft–CyA vs Allograft+pCyA, Autograft – CyA & Isograft +/- CyA
**p<0.05	Allograft+oCyA vs Autograft – CyA & Isograft +/- CyA
• p<0.05	Autograft+CyA vs Autograft-CyA, Isograft+CyA
•• p<0.05	Autograft-CyA vs Allograft+pCyA

One way ANOVA to compare groups (p<0.001). Tukey multiple comparison procedure to compare every pair of group means.

Table 6.4

21 day Percentage area of axonal staining – Proximal Graft Mean (+/-SD), n=6

	Allograft	SandAllo	SandControl
+pCyA	19.66* (+/-1.79)	14.94 (+/-1.47)	
+oCyA	14.13 (+/-4.14)		
-CyA	11.89 (+/-4.66)	14.84 (+/-2.18)	10.75 (+/-2.46)

• p<0.05	Allograft+pCyA vs Allograft-CyA, Allograft+oCyA, SandControl

One way ANOVA to compare groups (p<0.001). Tukey multiple comparison procedure to compare every pair of group means.

These results indicate that while oily CyA is associated with an improvement in axonal regeneration within an allograft model, the benefit is not as great as that conferred by parenteral CyA.

6.4.1.2 Macrophage Quantification

Analysis of macrophage staining (Tables 6.5) also indicates that oily preparation CyA produces results that are part-way between those of parenteral CyA and the non-immunosuppressed allografts. The Allo+oCyA group results were more variable than the other groups. Although, unlike the Allo+pCyA group, they show statistically increased macrophage staining compared to isograft controls (Table 6.6).

Table 6.5

21 day Percentage area of macrophage staining – Proximal Allograft Mean (+/-SD), n=6

	+pCya	+oCya	-CyA
Allograft	17.11* (+/-4.60)	25.26 (+/-7.72)	30.26 (+/-2.99)

* $p < 0.05$ Allograft+pCyA vs Allograft-CyA

One way ANOVA to compare groups (p=0.003). Tukey multiple comparison procedure to compare every pair of group means.

Table 6.6

21 day Percentage area of macrophage staining – Proximal Graft Mean (+/-SD), n=6

	Allograft	Autograft	Isograft	SandAlloG
+pCyA	17.11 (+/-4.60)	18.95 (+/-6.73)	12.35 (+/-2.19)	10.99*** (+/-1.75)
+oCyA	25.26** (+/-7.72)			
-CyA	30.26* (+/-2.99)	21.30 (+/-2.66)	15.80 (+/-2.39)	18.17 (+/-4.32)

* p<0.05	Allograft-CyA vs Allograft+pCyA, Autograft+pCyA, SandAlloG+/-CyA, Isograft+/-CyA
**p<0.05	Allograft+oCyA vs SandAlloG+pCyA, Isograft+/-CyA
***p<0.05	SandAlloG+pCyA vs Autograft-CyA

One way ANOVA to compare groups (p<0.001). Tukey multiple comparison procedure to compare every pair of group means.

Comparison with sandwich grafts indicates similar levels of staining to the non-immunosuppressed sandwich allograft, but higher levels than within the SandAllo+pCyA group. All groups receiving parenteral CyA had lower levels of staining than their non-immunosuppressed counterparts. This implies that, for allogenic graft segments, both administration of oily CyA and addition of the autologous graft segment within the sandwich graft construct lead independently to a similar reduction in the macrophage response. Administration of seemingly more potent parenteral CyA enhances this response, producing similar amounts of macrophage staining to that measured in non-allogenic grafts.

6.4.1.3 Schwann Cell Quantification

Comparison of Schwann cell staining within the early graft groups indicates greater Schwann cell quantification within the Allo+pCyA group. Results from the Allo+oCyA group lie between its allogenic counterparts, although on this occasion differences between both groups are significant. Schwann cell staining within the Allo+oCyA group is also poorer than within the non-allogenic groups but remains significantly better than the Allo-CyA group.

When compared to Sandwich graft models (Table 6.8), the Allo+oCyA group results were similar to the allogenic sandwich graft groups.

Table 6.7

21 day Percentage area of Schwann cell staining – Proximal Graft Median (25-75%), n=6

	Allograft	Autograft	Isograft
+pCyA	41.86 (31.91-49.66)	38.63 (35.62-40.94)	37.54 (36.07-40.67)
+oCyA	25.14** (7.63-32.79)		
-CyA	9.94* (4.39-11.19)	45.56 (42.55-47.84)	47.59 (46.02-49.95)

* p<0.05 Allograft–CyA vs All groups
**p<0.05 Allograft+oCyA vs All groups

One way ANOVA on ranks to compare groups (p<0.001). Student-Newman-Keuls multiple comparison procedure to compare every pair of group medians.

Table 6.8

21 day Percentage area of Schwann cell staining – Proximal Graft Mean (+/-SD), n=6

	Allograft	SandAllo	SandControl
+pCyA	40.92 (+/-9.69)	31.45 (+/-5.70)	
+oCyA	21.69 (+/-12.42)		
-CyA	8.30 (+/-4.55)	28.04 (+/-8.62)	41.16 (+/-6.69)

* $p<0.05$ Allograft-CyA vs Allograft+pCyA, All sandwich graft groups.
** $p<0.05$ Allograft+oCyA vs Allograft+pCyA, SandControl.

One way ANOVA to compare groups ($p<0.001$). Tukey multiple comparison procedure to compare every pair of group means.

6.4.2 Long-term regeneration - 32 weeks

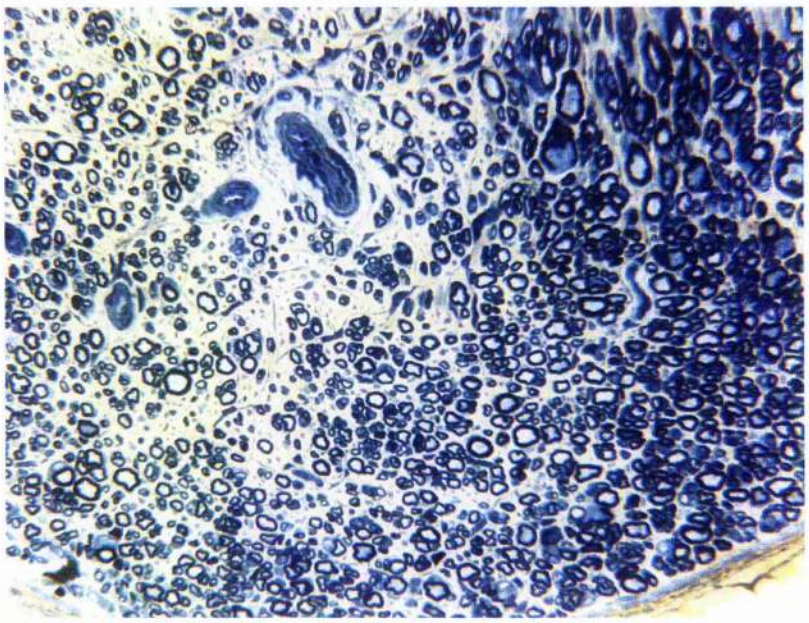
6.4.2.1Myelinated axon analyses

Analyses for long-term results were undertaken on semithin sections of posterior tibial nerve using measurements taken from myelinated axons, as described in Chapters 2 and 5 (Figure 6.1).

Total axon counts for allograft and sandwich allograft groups immunosuppressed with parenteral CyA (Allo+pCyA and SandAllo+pCyA) show lower results with narrower variability than the corresponding sandwich and allograft groups (Table 6.9).

Figure 6.1 Semithin sections of distal myelinated nerve at 32 weeks

Allo+pCyA x 40



SandAllo+pCyA x 40

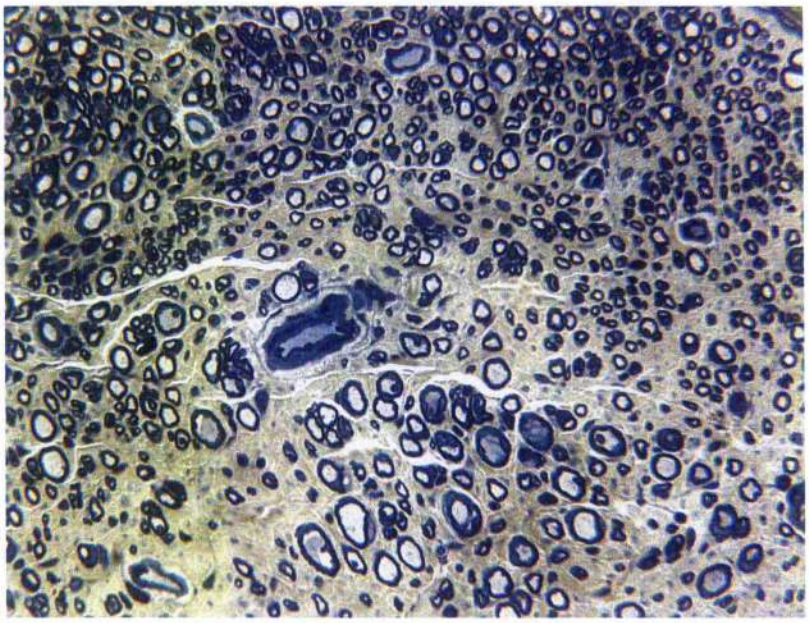


Table 6.9 Total Axon Counts in 3 Frames – Mean +/- SD

Group	n	CyA	Type	No. Axons	SD
Simple Grafts					
Allograft	6	yes	oCyA	1251	(+/-262)
Allograft	5	yes	pCyA	1035	(+/-146)
Allograft	6	no		1274	(+/-330)
Isograft	5	yes	oCyA	1062	(+/-214)
Isograft	5	no		903	(+/-484)
Sandwich Grafts					
SandAllo	5	yes	oCyA	998	(+/-305)
SandAllo	6	yes	pCyA	704 *	(+/-184)
SandAllo	6	no		1338	(+/-359)
SandCon	6	yes	oCyA	949	(+/-304)
SandCon	6	no		1145	(+/-96)
Controls					
Normal	6	no		505	(+/-55)
Unrepaired	5	no		12	(+/-5)
Allo+vehicle	6	no		1279	(+/-683)

*$p<0.05$ for SandAllo+pCyA vs Allo+oCyA, Allo-CyA, SandAllo-CyA

One way ANOVA comparing Simple (including Allo + vehicle control) with Sandwich grafts ($p=0.004$). Tukey multiple comparison procedure comparing every pair of group means.

When compared with matching non-allogenic groups, only the SandAllo+pCyA group shows any significant difference with lower values than the Allo+oCyA, Allo-CyA and SandAllo-CyA groups. Normal and unrepaired groups have been shown for completeness but were uninvolved in this analysis.

When the Normal group was compared to the experimental groups (Table 6.10) results from Allo+pCyA and SandAllo+pCyA were similar to normal values.

Table 6.10 Total Axon Counts in 3 Frames – Median values

Group	n	CyA	Type	No. Axons	(25-75 centiles)
Simple Grafts					
Allograft	6	yes	oCyA	1137	(1068-1508)
Allograft	5	yes	pCyA	1011	(968-1125)
Allograft	6	no		1235	(931-1583)
Isograft	5	yes	oCyA	1035	(929-1205)
Isograft	5	no		932	(721-1104)
Sandwich Grafts					
SandAllo	5	yes	oCyA	1125	(801-1222)
SandAllo	6	yes	pCyA	679	(582-777)
SandAllo	6	no		1221	(1069-1760)
SandCon	6	yes	oCyA	926	(755-1138)
SandCon	6	no		1184	(1087-1216)
Controls					
Normal	6	no		526 *	(474-593)
Unrepaired	5	no		11	(8-15)
Allo+vehicle	6	no		1129	(896-1664)

Comparing the Normal control to all other groups (except Unrepaired), Kruskal-Wallis One way ANOVA on ranks executed ($p=0.001$). Dunn's multiple comparison procedure, *$p < 0.05$ Normal vs Allograft+oCyA, Allograft-Cya, SandAllo-CyA, SandControl-CyA.

Results from Unrepaired nerve were omitted from statistical analysis as gross difference and variability skewed analyses, results included in table to indicate this.

Axon and fibre diameter measurements also indicated larger fibres within the Allo+pCyA and SandAllo+pCyA groups compared to the other experimental groups. However, these results were not statistically different (Table 6.11). All groups were statistically thinner than normal nerve, but when compared to unrepaired nerve, the values from the Allo+pCyA and SandAllo+pCyA groups reached statistical significance. For fibre diameter, the Allo+oCyA, Iso+oCyA and SandAllo-CyA groups were also significantly better than axons within the unrepaired group.

Table 6.11 Myelinated Nerve Morphology – Mean (+/- SD)

Group	n	CyA	Type	Axon Diameter μm	Fibre Diameter μm
Simple Grafts					
Allograft	6	yes	oCyA	1.78 (+/-0.14)	4.47 (+/-0.25)
Allograft	5	yes	pCyA	2.20 (+/-0.17)	4.62 (+/-0.20)
Allograft	6	no		1.76 (+/-0.37)	4.21 (+/-0.44)
Isograft	5	yes	oCyA	1.99 (+/-0.39)	4.55 (+/-0.54)
Isograft	5	no		1.69 (+/-0.26)	4.29 (+/-0.37)
Sandwich Grafts					
SandAllo	5	yes	oCyA	1.97 (+/-0.26)	4.42 (+/-0.32)
SandAllo	6	yes	pCyA	2.40 (+/-0.34)	4.94 (+/-0.49)
SandAllo	6	no		1.75 (+/-0.20)	4.42 (+/-0.29)
SandCon	6	yes	oCyA	1.72 (+/-0.26)	4.28 (+/-0.26)
SandCon	6	no		1.63 (+/-0.20)	4.38 (+/-0.38)
Controls					
Normal	6	no		4.70 (+/-0.48) *	8.39 (+/-0.62) *
Unrepaired	5	no		1.24 (+/-0.27) **	3.13 (+/-0.34) **
Allo+vehicle	6	no		1.87 (+/-0.35)	4.29 (+/-0.51)

Comparing **Axon diameters** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups; **$p<0.04$ Unrepaired vs Allograft+pCyA, SandAllo+pCyA.

Comparing **Fibre diameters** One Way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups; **$p<0.04$ Unrepaired vs Allograft+oCyA, Allograft+pCyA, Isograft+oCyA, SandAllo+pCyA, SandAllo-CyA.

Myelin thickness calculation indicates no differences between the experimental groups, although all were thinner than normal (Table 6.12). The results from G-ratio analysis show similar findings except that the higher levels recorded for the parenteral CyA groups were statistically similar to normal nerve values. Comparisons with SandAllo-CyA and SandControl groups also showed the Allo+pCyA and SandAllo+pCyA groups to be significantly better. Analysis of laciness indicated mainly similarities between the groups, with only the difference between SandAllo+pCyA and SandAllo-CyA reaching statistical significance.

Table 6.12 Myelinated Nerve Morphology – Mean (+/- SD)

Group	n	CyA	Type	Myelin Thickness (μm)	G-ratio	Laciness
Simple Grafts						
Allograft	6	yes	oCyA	1.35 (+/-0.10)	0.34 (+/-0.03)	0.85 (+/-0.01)
Allograft	5	yes	pCyA	1.22 (+/-0.06)	0.42 (+/-0.02)	0.84 (+/-0.01)
Allograft	6	no		1.22 (+/-0.08)	0.36 (+/-0.04)	0.84 (+/-0.03)
Isograft	5	yes	oCyA	1.28 (+/-0.12)	0.38 (+/-0.04)	0.84 (+/-0.02)
Isograft	5	no		1.30 (+/-0.06)	0.34 (+/-0.03)	0.82 (+/-0.03)
Sandwich Grafts						
SandAllo	5	yes	oCyA	1.23 (+/-0.06)	0.37 (+/-0.02)	0.84 (+/-0.03)
SandAllo	6	yes	pCyA	1.27 (+/-0.16)	0.42 (+/-0.04)	0.85 (+/-0.02)
SandAllo	6	no		1.34 (+/-0.09)	0.33 (+/-0.03) *	0.79 (+/-0.01)***
SandCon	6	yes	oCyA	1.28 (+/-0.05)	0.34 (+/-0.03)	0.83 (+/-0.02)
SandCon	6	no		1.38 (+/-0.12)	0.32 (+/-0.03) **	0.83 (+/-0.03)
Controls						
Normal	6	no		1.85 (+/-0.09) *	0.50 (+/-0.01) **	0.85 (+/-0.02)
Unrepaired	5	no		0.58 (+/-0.48)	0.26 (+/-0.22)	0.51 (+/-0.42)
Allo+vehicle	6	no		1.21 (+/-0.14)	0.39 (+/-0.05)	(+/-)

Comparing **Myelin Thickness** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.

Comparing **G-ratio** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, **$p<0.002$ Normal vs all groups, except Allograft+pCyA and SandAllo+pCyA. *$p<0.05$ SandAllo-CyA vs Allograft+pCyA, SandAllo+pCyA. **$p<0.02$ SandCon-CyA vs Allograft+pCyA, SandAllo+pCyA.

Comparing **Laciness** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, ***$p=0.041$ SandAllo-CyA vs SandAllo+pCyA

Unrepaired group omitted from statistical analysis in view of gross difference and high variability, results included in table to indicate this.

The distribution of myelinated fibres within the groups identified no statistical differences between the experimental groups within the low, middle or high fibre size ranges (Table 6.13). All groups were however statistically different to normal nerve with regenerated fibres mainly being of small calibre. Comparison with results from unrepaired nerve only reached statistical significance for the Sand Allo+pCyA group. Within the experimental groups, this group produced the largest amount of thick fibres.

Table 6.13

Frequency Distribution of Fibre Diameters – Mean Percentage +/- SD

Groups	CyA	Type	% Small 1-7μm	% Medium 8-10μm	% Large 11-20+μm
Simple Grafts					
Allograft	yes	oCyA	87.77 (+/-2.73)	9.42 (+/-2.04)	2.80 (+/-0.84)
Allograft	yes	pCyA	87.32 (+/-1.96)	10.35 (+/-1.69)	2.33 (+/-0.74)
Allograft	no		90.76 (+/-5.66)	7.63 (+/-4.22)	1.61 (+/-1.47)
Isograft	yes	oCyA	87.00 (+/-7.08)	9.98 (+/-4.65)	3.03 (+/-2.81)
Isograft	no		90.13 (+/-3.73)	8.15 (+/-2.90)	1.72 (+/-0.85)
Sandwich Grafts					
SandAllo	yes	oCyA	86.51 (+/-5.03)	10.23 (+/-3.15)	3.27 (+/-2.14)
SandAllo	yes	pCyA	81.77 (+/-6.01)	12.83 (+/-3.44)	5.40 (+/-3.73)
SandAllo	no		87.48 (+/-4.47)	9.55 (+/-2.91)	2.97 (+/-1.63)
SandCon	yes	oCyA	87.87 (+/-2.87)	9.24 (+/-1.97)	2.89 (+/-1.49)
SandCon	no		88.32 (+/-4.95)	9.26 (+/-2.98)	2.42 (+/-2.09)
Controls					
Normal	no		33.39 * (+/-4.35)	31.56 * (+/-5.12)	35.05 ** (+/-9.33)
Unrepaired	no		98.00 ** (+/-4.47)	2.00 (+/-4.47)	0.00 (+/-0.00)
Allo+vehicle	no		90.30 (+/-5.17)	8.06 (+/-3.85)	1.64 (+/-1.36)

Comparing **% Small** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups; **$p=0.024$ Unrepaired vs SandAllo+pCyA.
Comparing **% Medium** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, *$p<0.001$ Normal vs All groups.
Comparing **% Large** One way ANOVA ($p<0.001$), Scheffé multiple comparison procedure, **$p<0.001$ Normal vs All groups. However, omitting the Normal group improves the assumption of homogeneous variances. Subsequent One way ANOVA, $p=0.049$ showing no significant difference between the remaining groups.

6.4.2.2 Gastrocnemius muscle mass

Analysis of Gastrocnemius muscle mass from the experimental groups immunosuppressed with oily CyA and the non-immunosuppressed groups has been detailed in Chapter 5. Analysis of mass values for the groups immunosuppressed with parenteral CyA (Table 6.14) shows no statistical difference between groups, except that all groups had significantly improved muscle bulk to that recorded for the unrepaired nerve group.

Table 6.14

Gastrocnemius muscle mass – Percentage operated side / normal Median (25-75 centiles)

Group	n	CyA	Type	Median %	25-75 centiles
Simple Grafts					
Allograft	6	yes	oCyA	64.24	(53.98-70.90)
Allograft	5	yes	pCyA	65.27	(63.32-71.98)
Allograft	6	no		68.21	(59.05-72.93)
Isograft	5	yes	oCyA	58.91	(52.27-63.52)
Isograft	5	no		49.29	(35.62-59.40)
Sandwich Grafts					
SandAllo	5	yes	oCyA	65.00	(61.37-65.67)
SandAllo	6	yes	pCyA	64.23	(62.44-67.11)
SandAllo	6	no		72.39	(69.91-74.69)
SandCon	6	yes	oCyA	68.20	(60.23-75.42)
SandCon	6	no		67.75	(65.63-68.61)
Controls					
Unrepaired	5	no		11.68 *	(9.69-13.11)
Allo+vehicle	6	no		66.56	(51.83-74.68)

Kruskal-Wallis One way ANOVA on ranks executed using Median values.

Comparing Unrepaired to experimental grafts (including Allograft+vehicle), K-W ANOVA, $p \leq 0.002$. Dunnett's multiple comparison procedure, *$p<0.05$ Unrepaired vs All groups.

Omitting Unrepaired group, K-W ANOVA shows no significant difference between the remaining groups, $p=0.061$.

The use of parenteral formulation CyA (pCyA) appears to have some benefit over oily CyA (oCyA) over long and short term experimental periods when comparing regeneration parameters. However, in the majority of cases the measured differences do not reach statistical significance. The extent of regeneration found within experimental groups was greatly improved from that seen in an unrepaired identical model.

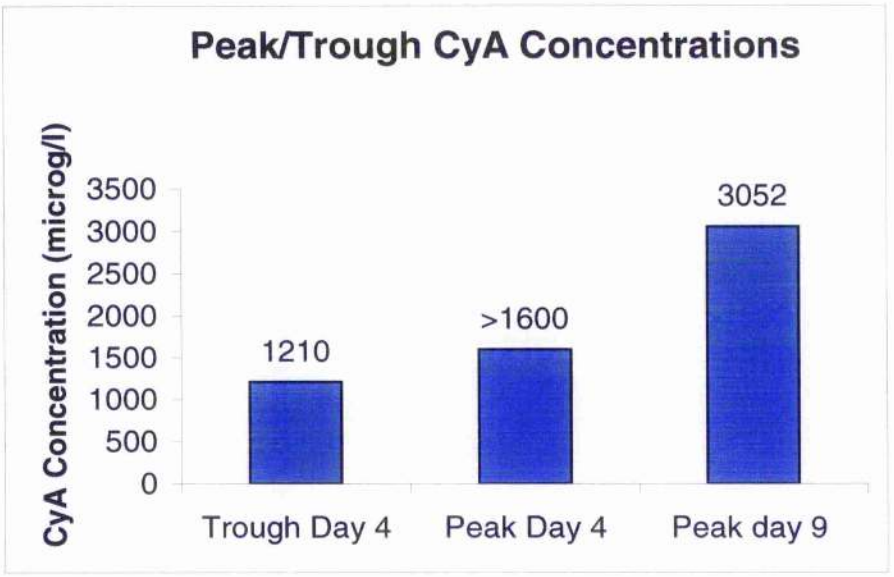
6.4.3 Cyclosporin monitoring

As improved levels of immunosuppression with general methods are often associated with increased drug toxicity and side-effects, for these experiments evaluation of CyA, creatinine, and liver enzymes blood levels were measured, along with the animals' weights.

6.4.3.1 Cyclosporin levels

Assessments of CyA levels measured pre- and 4 hours post-dose indicate that levels were satisfactory following drug administration (Figure 6.2) and agree with other reported levels [213,262] . The highest levels were associated with parenteral formulation CyA. Unfortunately within the biochemistry laboratory carrying out the analyses concentrations greater than 1500µg/l were often reported as such and not as absolute values, therefore no statistical analysis of these results was possible, as results would have been meaningless.

Figure 6.2



Trough CyA concentrations were measured from blood samples obtained from the relevant groups at weeks 12 and 32 during the long-term (32 week) experiments (see Chapter 2 for methods). At 12 weeks, analysis of specimens from the Allo+oCyA and SandAllo+oCyA groups showed no significant differences in trough CyA levels 24 hours following the last CyA dose (Table 6.15).

At 32 weeks, specimens were analysed from groups receiving oily and parenteral CyA. Comparisons within the oily CyA groups showed similar trough values (Table 6.15). These were significantly higher than levels recorded at 12 weeks. Results from the parenteral CyA groups were lower than for oily CyA groups. The Allo+pCyA and SandAllo+pCyA groups produced vastly different mean levels. The reason for this, as determined from the experimental protocol diary may be that over 48 hours had elapsed following the last dose to the SandAllo+pCyA group compared to 24 hours for the Allo+pCyA group. The SandAllo+pCyA group would usually have had CyA but it was omitted as the group was due for harvest,

however this low level could also represent a laboratory based fault or a spurious finding.

Table 6.15

Blood Cyclosporin Concentration - Mean (+/-SD)

Group	n	CyA type	Trough levels (μg/l) 12 weeks	32 weeks
Simple Grafts				
Allograft	6	oCyA	340.17** (+/-108.84)	660.33 (+/-144.45)
Allograft	5	pCyA		245.17)* (+/-12.45)
Isograft	5	oCyA		486.60 (+/-157.09)
Sandwich Graft				
SandAllo	5	oCyA	315.60** (+/-57.24)	645.83 (+/-200.75)
SandAllo	6	pCyA		40.00 (+/-11.58)

One way ANOVA comparing CyA levels in groups receiving oCyA at 32 weeks (p=0.16).

K-W ANOVA on ranks comparing groups receiving oCyA and pCyA at 32 weeks (p=0.002). SandAllo+pCyA omitted. Dunn's multiple comparison procedure *p<0.05 Allo+pCyA vs Allo+oCyA, SandAllo+oCyA.

t-test comparing CyA levels in the 2 groups receiving oCyA at 12 weeks (p=0.662).

One way ANOVA comparing groups receiving oCyA at 12 and 32 weeks (p<0.001). Tukey multiple comparison procedure, **p<0.05 All 32 week oCyA groups vs All 12 week oCyA groups.

These results suggest a depot effect was present within this model for the subcutaneous route of CyA administration, but that the depot effect was mainly associated with the oily CyA preparation.

6.4.3.2 Hepatic and renal function

Measurements of plasma concentrations of creatinine, alkaline phosphatase, AST and ALT are shown in Table 6.16. In the isograft group, all of the specimens were haemolysed and analysis could not be conducted on them (Haem).

For creatinine and alkaline phosphatase, the mean levels from every group are within the upper reference range used by the laboratory undertaking the analyses (Guy's Hospital Biochemistry Dept. – Chapter 2). Likewise, ALT levels are within the range, with only the Allo+pCya group being just outside. The AST levels are, however, much higher than expected. As the laboratory reference values relate to human blood samples, blood was taken from 28 non-immunosuppressed groups at the end of the experiment to be used to determine rat control concentrations for the substances under investigation. These values are reported as *Rat Controls* within Table 6.16.

Comparison with the *Rat Controls* indicates that the lower values for creatinine are probably normal for these rats. Creatinine concentration relates to muscle mass, therefore rat levels would be expected to be lower than human. Also the levels of Alkaline phosphatase and ALT appear normal for these rats. While there were no statistical differences between Controls and experimental groups in the creatinine and ALT groups, the Iso+oCyA and Allo+pCyA groups were significantly lower than the control value for alkaline phosphatase. However it is unlikely that this difference has any relevance in real terms in so much as it is low and CyA toxicity is associated with high levels.

Table 6.16 Creatinine, Alkaline Phosphatase, AST, ALT Blood Levels

Group	n	CyA Type	Creat (μmol/l)	AlkPhos (iu/l)	AST (Iu/l)	ALT (Iu/l)
Simple Grafts						
Allograft	6	oCyA	40.5 (+/-4.93)	76.17 (+/-17.31)	295.5 (+/-68.85)	40.33 (+/-15.97)
Allograft	6	pCyA	42.67 (+/-1.63)	55.17 (+/-8.50)	256.33 (+/-39.37)	59.83 (+/-12.81)
Isograft	5	oCyA	Haem	47.60* (+/-8.17)	Haem	51.80 (+/-4.97)
Sandwich Graft						
SandAllo	6	oCyA	40.67 (+/-4.08)	79.17 (+/-26.31)	332.5 (+/-57.58)	39.00 (+/-21.40)
SandAllo	5	pCyA	38.8 (+/-2.68)	83.00 (+/-10.79)	281 (+/-70.70)	40.80 (+/-23.84)
Rat Controls		**none**	**40.87 (+/-13.96)**	**117.61** (+/-79.54)**	**174.76 (+/-89.67)**	**61.54 (+/-59.57)**
Human Ref. Values			**65-101**	**38-126**	**0-35**	**0-55**

Creatinine K-W ANOVA on ranks comparing experimental groups (p=0.13): no significant differences. No difference compared to Control Creatinine (p=0.309).

Alk Phos One Way ANOVA comparing experimental groups (p=0.004). Tukey multiple comparison procedure: *p<0.05 Iso+oCyA vs SandAllo+oCyA and pCyA. Compared to Control Alkaline Phosphatase, **p<0.05 Iso+oCyA and Allo+pCyA vs Control.

AST K-W ANOVA on ranks comparing experimental and control groups (p<0.001). Dunn's multiple comparison procedure, p<0.05 SandAllo+oCyA vs AST Control.

ALT One Way ANOVA comparing experimental groups (p=0.197): no significant difference. No difference compared to Control ALT (p=0.518).

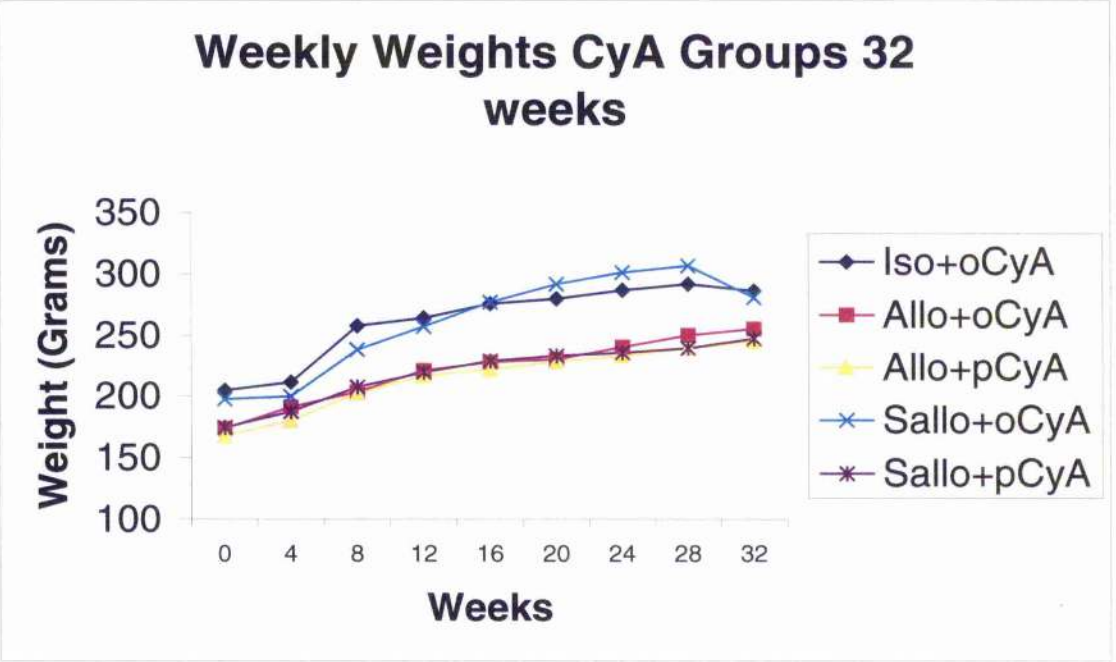
Haem: Haemolysed samples.

Regarding AST measurement, when compared to *Rat Control* values, only the SandAllo+oCyA produced significantly higher levels of AST. All other groups, although recording high concentration, may have to be regarded as normal within anaesthatised Lewis rats.

6.4.3.3 Weight gain

Groups receiving CyA were weighed weekly, to determine the dose of CyA to administer and also as a monitor of general health (Figure 6.3). Non-immunosuppressed groups were weighed at the start and end of the experiments (Figure 6.4).

Figure 6.3



Starting Weight	One Way ANOVA comparing groups ($p<0.001$). Tukey multiple comparison procedure: *$p<0.05$ Iso+oCyA and SandAllo+oCyA vs All other groups.
Final Weight	Similar findings to above.

All animals gained weight whether immunosuppressed or not. In the immunosuppressed groups, the graph of weight gain plotted over four weekly increments identifies two distinct populations. Statistical analysis identifies that this

difference is related to the different mean starting weights of these groups but generally the rate of weight gain was similar between all groups (Table 6.17). The rate of weight gain was greatest within the Normal group but this also did not reach statistical significance (Figure 6.4 and Table 6.17). From this we can conclude that as far as this parameter is concerned, CyA administration did not appear to have a detrimental effect on the rats' general health, any more so than the operative procedure itself.

Figure 6.4

Table 6.17 Mean Rate of Group Weight Gain over 32 Weeks

Simple Grafts	Rate(g/week)	Sandwich Grafts	Rate(g/week)
Iso-oCyA	2.46	SandCon-CyA	2.40
Iso+oCyA	2.32		
Allo-CyA	2.56	SandAllo-CyA	2.90
Allo+oCyA	2.48	SandAllo+oCyA	2.62
Allo+pCyA	2.42	SandAllo+pCyA	2.28
Normal	3.30		

One Way ANOVA comparing experimental groups (p=0.843): no statistical difference. Comparison with Normal also non-significant (p=0.159).

6.4.4 Morbidity and Mortality

There were nine unexpected deaths during this project. The majority were associated with general anaesthesia. These numbers include two rats who underwent euthanasia in the early stage of the project following prolonged and inadequate post-operative recovery despite standard anaesthetic precautions and recovery room practices, as per veterinary advice and Home Office regulations. As female rats were used in these experiments, it was felt that the temperature of the post-operative recovery room may have been inadequate for recovering these rats. Other experiments in the same facility used mainly male rats which are much larger than females of the same age. Following an increase in recovery room temperature, along with the provision of subcutaneous saline already being practiced, further preoperative deaths were prevented.

Two further rats required euthanasia. Both belonged to long-term experimental groups receiving oily CyA. One developed sudden onset left hind limb paralysis at 30 weeks (Allo+oCyA), while the other developed a middle ear infection (Iso+oCyA). The former rat had no evidence of infection and appeared to have had

a CVA (cerebrovascular accident), while the latter rat's death could be related to CyA administration because of its infective nature.

One other unexpected death was attributed to CyA. This long-term experimental animal was receiving daily oily CyA within the first 12 weeks of the experiment. The animal had been noted to have developed a soft non-tender scruff swelling (see later) which was thought to be related to CyA administration. This animal was found dead. Post mortem findings noted cloudy oily fluid within the scruff subcutaneous swelling which grew Staphlococcus aureus on bacteriological culture. All other organs appeared normal. The death was therefore attributed to septicaemia, secondary to infection of a collection of oil based CyA preparation. No cage mates became unwell.

Morbidity within the experimental groups fell into four main groups: autoamputaton, scruff subcutaneous fluid collections, cutaneous pressure effects and rashes.

Two animals had autoamputated toes within three weeks of denervation. Both animals were not immunosuppressed and both injuries involved lateral right toes (3 in one rat and 1 in the other rat). The animals belonged to short and long-term experiments respectively. Three rats had right footpad skin changes, which included a 2mm dry, healing ulcer, a small granuloma which healed and a keratin horn at the base of the fifth metatarsal. Neither of these animals were immunosuppressed. Rats from four groups had skin rashes which produced a few dry scaling spots within the coats on the main corporeal parts of the animals. Fourteen animals out of a possible 24 were affected, all within long-term groups receiving CyA, irrespective of the formulation. There was no evidence of infection or infestation. All the rashes were noted towards the end of the experiment therefore no comment can be made about the long-term prognosis of this condition

but within the confines of the experiment the animals continued to behave normally and gain weight while affected by the rash.

Ten animals, mainly from two groups were noted to have developed scruff subcutaneous swellings towards the end of the experiment although a few were noted from as early as six weeks into the long-term experiments. All of the groups were receiving oily CyA. The injection sites for CyA administration were varied to try to avoid any drug accumulation and tissue reaction, however in the small female rat inevitably the scruff area appeared to have been a favoured site. A coalescent effect from injections appeared to produce these soft, fluid, oily collections of a few millilitres in volume. None of the animals appeared distressed by the collections although one death was attributed to infection of such a collection (see above). To avoid causing further problems, following discovery of a collection, the whole scruff area was avoided for drug administration and to prevent the introduction of potential pathogens. For this reason the collections were not drained electively.

Therefore for the whole project, from 12 deaths, two may have been associated with CyA, and out of 29 animals with noted morbidity, 24 were associated with CyA use. The vast majority of animals had no decernable problems.

6.5 DISCUSSION

Results from Chapter 5 indicate no significant difference in the long term regeneration potential of simple or sandwich allografts irrespective of immunosuppression following repair of a 10mm sciatic nerve defect with 15mm grafts. Indeed, results from both nerve graft types were similar to controls. However, while all fell short of normal sciatic nerve in terms of myelinated axon morphology and gastrocnemius muscle mass, all groups produced significantly

improved regeneration than that of an unrepaired nerve defect. Based on these findings, the question remained as to whether or not the oily formulation of CyA being used in Chapter 5 was providing adequate immunosuppression.

To assess the efficacy of oCyA and pCyA further experiments were undertaken using oCyA in the short term and pCyA in the long term so that these additional groups could be compared with those already reported in Chapters 3 and 5.

Against a background of some resistance to clinical nerve allografting on ethical grounds, additional assessments were made to determine any undesired effects of CyA that may also be influential in future decision making regarding nerve allografting.

The efficacy of any drug relates to its bioavailability in sufficient concentration to produce the desired effects while minimising any negative effects in any individual recipient. Cyclosporin is poorly water soluble [198,200,202] and although delivery orally in humans is a major benefit, in small animals the oral route has been associated with highly variable bioavailability [213] . The provision by Sandoz of base powder CyA has meant many authors have used this prepared in oil and alcohol as undertaken here. Wasseff, Bain and Midha have contributed important information on the use of CyA in rat models [206,213,254,262] . From their work, although higher doses were associated with greater bioavailability more pronounced immunosuppression, a dose of 5mg/kg/day was the minimum required to maintain satisfactory immunosuppression [206,213] with this dose Bain showed total suppression of a mixed lymphocyte reaction. With this dose equivalent to syngeneic controls while side effects were minimised. In a rat model incompatible at both MHC I and II loci [206] . The same administration route and does were subsequently used in a peripheral nerve allograft study where at 14 weeks regeneration in an immunosuppressed PNAG was equivalent to the autograft

controls [207] . Midha however also demonstrated with subcutaneous delivery of CyA that a depot effect was evident secondary to fat binding [254] . If overlooked this could result in higher levels of circulating drug than expected with a greater risk of toxicity. Beneficial effects of a depot effect mean that reduced drug dosing is required while maintaining steady-state circulating drug concentrations. This minimises toxicity associated with gross concentration fluctuations. The use of an oil based CyA formulation in the long-term experiments (see Chapter 5) was suspected to produce such a depot effect and to beassociated with poorer bioavailability. The development of subcutaneous fluid connections provided visual evidence that this may be occurring [262] , therefore the decision to reduce dosage interval during the experimental period appears justified, provided a steady state has been reached.

Axonal regeneration described in Chapter 3 for simple allografts immunosuppressed with pCyA was equivalent to that in non-allogenic control nerves. Associated with this were findings of reduced macrophage infiltration and greater Schwann cell presence than that identified in the non-immunosuppressed allografts. The administration of oCyA to the same model in the same dose produced effects midway between those seen in the non-immunosuppressed and pCyA groups. However the beneficial effects of CyA were evident when compared with the same parameters measured from non-immunosuppressed nerve allografts. This indicates poorer efficacy of oCyA compared to pCyA which likely relates to poorer bioavailability of oCyA related to its reduced solubility.

Results of long term studies using both CyA formulations indicates no significant difference in outcome across morphological parameters or related to the restoration of muscle mass with reinnervation. The lower myelinated axon counts, approaching normal values, associated with pCyA administration and the higher values across other paramenters may, despite the lack of statistical significance indicate greater potential benefits with pCyA use.

As indicated in Chapter 5, the interpretation of axon counts in isolation is difficult. It is understood that during regeneration greater numbers of smaller axons regenerate following injury than the numbers measured in normal nerve [3,52] . Once distal connections are established "pruning" occurs [325] . However the residual axon counts remain in excess of normal nerve axon counts although function does not reach normality implying some of these axon are inappropriately connected [327] . Therefore, conclusions regarding adequacy of axonal regeneration long term are related to comparisons made with results obtained from the standard repair methods of grafting or direct coaptation. In Chapter 5 and here, axon counts were increased compared to normal. However, when other parameters were also considered, particularly for the pCyA groups, the lower axon counts are suggestive that this indicates a beneficial response.

Despite the evidence from early regeneration periods, the similarity of outcomes from long-term regeneration could still be interpreted as secondary to the ineffective action of CyA. Therefore circulating CyA concentrations were measured as is regularly undertaken for monitoring purposes in clinical transplantation. The Biochemistry department used here to measure CyA had reference values for trough CyA concentrations of 150μg/l for renal and 300-400μg/l for cardiac and hepatic allografts.

Unlike adult humans, venopuncture is difficult in small animals and also only small volumes can be removed for testing without the animal experiencing negative effects. Therefore in these experiments regular monitoring of blood CyA levels was deemed inappropriate. Generally blood was taken at the end of the experiment along with the main specimen retrieval. This allowed larger volumes to be removed for tests of renal and hepatic functions.

In the early phases of this project some animals did have peak and trough blood CyA levels analysed (Figure 6.3 and Table 6.15). These results indicate that satisfactory trough levels were achieved when compared to the reference values given above. Results from trough levels of oCyA at 12 and 32 weeks also appear satisfactory and increase throughout the time of the experiment. These findings agree with those of Midha regarding a depot effect developing from subcutaneous CyA administration which was beneficial in maintaining steady-state CyA concentration using alternate day administration [254] . Taken in conjunction with peak levels, knowledge of suitable levels for solid organ transplantation and experience with the CyA used in these experiments, it could be argued that these measured levels could be reduced further without losing immunosuppression once a steady state has been achieved. However, as the study of CyA pharmacokinetics was not the aim of this project, provided immunosuppression appeared satisfactory, the dosage regimen was unaltered.

The greater fluctuation in concentration witnessed for pCyA relates to its greater bioavailability associated with its greater solubility [255,256] . The greater the bioavailability, then also, the greater the drug clearance. Higher peaks and lower troughs are expected with parenteral administration as identified by Wassef using the intramuscular and intraperitoneal routes of administration [213] . In these experiments, parenteral formula CyA, although delivered subcutaneously also produced lower trough concentrations at 32 weeks than the oily preperation. This may explain the low CyA level measured for SandAllo+pCyA in Table 6.15, as animals due for CyA dosage in the morning but scheduled for harvest would not have received the CyA dose that morning therefore a maximum of 54 hours may have elapsed since their last dose.

Evidence from early nerve regeneration assessments indicates the dose of 5mg/kg/day produces adequate immunosuppression to allow axonal regeneration. Reducing the dosage interval avoids producing excessively high peak and trough

levels, which in turn will reduce the potential risk of drug toxicity [254] . To protect the regenerating axons up to, and including target organ reinervation the dosage interval was not reduced until 12 weeks had passed. This time point was also used by MacKinnon and Hare following their studies monitoring functional recovery by calculation of Sciatic Function Index [216,327] .

The majority of animals showed no signs of CyA toxicity although enough evidence has been accrued to indicate not insubstantial levels of morbidity.
Wasseff, Bain and Farthing all noted rat weight to be a useful indicator of well-being while receiving therapeutic doses of CyA [206,213,253] . Young rats (8 weeks) gain weight to reach adult proportions. This maturation was occurring during these experiments. The female Lewis rats used here were mainly less than 200g in weight at 8 weeks of age. The average weekly rate of weight gain was similar between experimental groups irrespective of CyA. For rats receiving CyA, two distinct populations were noted. Both collections of groups gained weight similarly and had initial and final weights consistently different. The slope of the graph appears to be reaching a plateau as the rats reached maturity. Although observation of the raw data indicates that individual rats occasionally did loose weight, the general trend overwhelmingly shows satisfactory and equivalent weight gain between groups. This in keeping with the findings of Bain and Wassef using the same does and route of administration [206,213] .

Levels of creatinine, alkaline phosphatase, AST and ALT were reasonably consistent within the experimental groups. In view of likely differences between rat and human values a number of control blood samples were taken from Lewis donor and experimental animals not receiving CyA. These blood samples were taken under identical conditions to those in the immunosuppressed rats. Only the results for AST were higher than expected. This may be normal for rats as indicated by the control blood samples. However could relate to the administration of general anaesthetic agents or to muscle damage associated with surgery and

cardiac-puncture for blood sampling. Farthing also noted this. Levels of AST from 28 control group rats ranged from 231 to 410 iu/l. These animals also had an inhalational anaesthetic. Farthing attributed these wide ranging levels to cardiac-puncturing. For his longer term experiment ALT instead. In that experiment there was no difference between control and experimental rat ALT levels regardless of CyA administration. The same was found here although the levels here were higher, on the other hand Farthings levels for alkaline phosphatase were higher. A significant rise in alkaline phosphatase was found using 25 mg and 50mg/kg after 10 days of administration. The lower results here are consistent with the low does CyA (5mg/kg) administered in this experiment. This does has been widely because it provides satisfactory immunosuppression with reduced risk of toxicity [206,213,254] .

There was some general morbidity recognised from animal observations related to CyA use. The most striking associations were skin rashes and subcutaneous fluid collections. This latter finding was exclusively related to oCyA administration. While neither of these conditions were widespread within the CyA groups, their association with the length of time of the experiment cannot exclude the potential risk of these problems becoming more widespread with time. Midha also experienced similar injection site problems which resolved spontaneously. He associated this with high ethanol concentrations used in the preparation of CyA solution [262] . Ethanol was also used to prepare CyA in these experiments. The small amount of morbidity related to right hind limb denervation was not unexpected. From the small numbers affected no conclusions can be made as to whether this could have been influenced by CyA administration or not.

As mentioned previously the majority of mortality was per-operative. Although attributed to general anaesthesia and the operative procedure, it cannot be ruled out from the data presented here that some contribution may have been made to mortality rates by CyA administration. These small female rats appeared less robust than their male counterparts (witnessed undergoing experiments in same

facility). These rats required maintenance subcutaneous fluid and a higher recovery room temperature than the male rats (GA is known to reduce ability to maintain homeostasis especially temperature). Add to this the development of high peak CyA concentrations in such susceptible animals and the risk of mortality could easily be increased.

While the majority of authors publishing research on experimental nerve allografting acknowledge the ethical dilema posed by immunosuppressant administration none supply much detail regarding their experiences of morbidity and mortality in their models other than that related to the nerve injury itself. However for human cases, MacKinnon with nerve allografts and Dubernard for upper limb transplantation site immunosuppressant associated morbidity leading to the cessation or alteration of immunosuppressant therapy [224,231] .

The most significant mortality associated with CyA in these experiments relates to infective episodes. Whether suddenly, or as euthanasia, two rats' deaths appeared related to infection. The scruff "abscess" case is the most clearly related death. Infection is also.associated with significant morbidity and mortality in human allotransplantation [104] .

Concerns regarding CyA and immunosuppressants generally have encouraged researchers to investigate the hypothesis borne from the knowledge that as axons are host derived, once through the nerve graft material, the conduit function may no longer be required. Immunosuppression could stop with subsequent rejection of the allograft, leaving the host axons. This frequently studied phenomenon has not delivered completely clear-cut results [97] . The timing of the duration of CyA administration has to be determined and the effect on ultimate regeneration established. The majority prefer target organ innervation to be underway or complete [158,209,216,238,267,325,327] . Using electrophysiology, morphology and

functional testing, an axonopathic process does follow immunosuppressant withdrawal. This is associated with donor Schwann cell loss and demyelination. However while some note no or incomplete recovery following the rejection assault [123,125,195,204,210,212,247], others report a full return of outcome indicators to pre CyA cessation levels [134,196,209,216,218,230].

Clinically, MacKinnon has the largest reported human series within the CyA era. She has had one failure due to subtherapeutic immunosuppression in seven cases over 10 years. FK506 has replaced CyA in the most recent cases. All of the patients were young (<24 years) and were immunosuppressed for a mean of 18 months with no significant loss of function following immunosuppressant withdrawal. Although recovery following these serious injuries is sub-optimal, the patients have retained limbs where salvage was debateable. These patients maintain protective sensation, and some can mobilise unaided and play sports [217,224,225].

The similarity between immunosuppressed and non-immunosuppressed groups for these long term experiments indicate little role for testing temporary immunosuppression within these models for either the simple or the sandwich graft procedures.

In summary, analysis of the different formulations of CyA used in this project indicate that both formulations were providing sufficient immunosuppression. Although oCyA appeared to provide lower levels of immunosuppression than pCyA in the short term, this difference was not significantly different as seen in the longer term experiment. However on balance pCyA was easier to administer with more predictable bioavailability and less associated morbidity, but because of its reduced capability of producing depot effects, its activity would benefit from more frequent monitoring and dose adjustment than oCyA. This would be practically easier in a

larger model, and essential to minimise morbidity and potential mortality in human allograft recipients.

CONCLUSIONS

Reported in this thesis are the results of experiments to determine whether the strategy of increasing the autologous Schwann cell complement within a peripheral nerve allograft (PNAG) model could improve the regenerative potential of host axons through PNAGs irrespective of immunosuppression.

Historically much research has sought to improve the abysmal results following neurological trauma. Function is best achieved following repair of transected single modality nerves and poorest following delayed repair of proximal mixed nerves where a structural defect exists. Local to the peripheral nervous system research effort has concentrated around identification and manipulation of the local environment and structures required for axonal regeneration. Many conduit materials have been proposed, some showing promise, but as yet, autologous nerve grafting remains the standard in clinical practice. The reason being that only nerve tissue satisfies all the criteria of an ideal conduit: the Schwann cell basal lamina scaffold containing supportive non-neuronal cells, especially Schwann cells. Unfortunately, the supply of expendable cutaneous nerves is limited and associated with donor site morbidity.

Allogenic nerve shares these ideal conduit components but its morbidity lies with immunological rejection and the side effects of immunosuppressants. Ethically, PNAG is viewed at best sceptically.

Immunosuppressed PNAGs are known to produce results similar to autografts. Advocates of PNAG have attempted to improve regeneration while minimising immunosuppressant requirements by a variety of strategies, including temporary

immunosuppression, rendering the grafts acellular and inducing donor specific tolerance. The latter has no clinical bearing and acellular conduits are generally inferior to autografts. The rationale for temporary immunosuppression is that axons are host derived, therefore once regeneration is established, the conduit function provided by the PNAG may no longer be required. However, results from temporary immunosuppression have produced conflicting results often related to methodology, while all agree that an axonopathic process follows immunosuppressant withdrawal.

Neuronal cell death secondary to trauma is partly responsible for diminished regeneration outcome. Early repair and restoration of neurotrophic influences are neuroprotective. Therefore a second assault from immunosuppressant withdrawal would be expected to negatively influence regeneration. For this reason, an "all or nothing" approach to immunosuppression was used here.

Schwann cells contribute to nerve antigenicity and in other acellular models have been shown to improve regeneration, whether inserted as nerve portions, cell cultures, or by the addition of the neurotrophic factors they produce. We hypothesized that by inserting a segment of autologous nerve into a nerve allograft, forming a chimeric structure of mixed antigenicity the depot of autologous Schwann cells would aid axonal regeneration by improving Schwann cell migration into the rejection PNAG. Also without immunosuppression, as allogenic Schwann cells are destroyed, repopulation with autologous Schwann cells will render the graft non-allogenic and reduce the delay period affecting regeneration with beneficial effects on target organ reinnervation.

Initial experiments assessed the early regenerative potential of non-immunosuppressed PNAG. Although poorer in terms of rate and amount of regeneration, the pattern of regeneration followed that of non-antigenic groups.

Increased macrophage staining and decreased Schwann cell staining were not witnessed in the immunosuppressed allografts group, indicating the poorer regeneration was related to an immunological response to allogenic Schwann cells.

Experiments using Sandwich allografts showed similar regeneration between groups regardless of immunosuppression and improved regeneration compared to simple non-immunosuppressed allografts. Schwann cells were qualitatively and quantitatively improved and macrophage staining reduced.

Long-term experiments further validated the use of Sandwich allografts. Assessment parameters were similar between all experimental groups. Neither lack of immunosuppression nor the extra neurorrhaphies appeared significant. While these results appeared to satisfy the aims and hypothesis of the thesis, they also raised some concerns regarding the project design, which have to be addressed before final conclusions can be drawn.

The rat strains employed were incompatible based on previous experience and evidence of rejection produced here. However, to further test the hypothesis comparing models of greater and lesser genetic disparity especially with larger defects requiring reconstruction would be beneficial and raise the chances of possible clinical application.

Two formulations of CyA were used in the project and both appeared to provide adequate immunosuppression. The parenteral CyA (pCyA) was associated with slightly improved results compared to the other groups. In view of the similarities in findings between groups, not only would use of a larger model make monitoring of immunosuppression simpler, but would also allow the Sandwich graft to be tested within larger reconstructions. Also repeating the experiments using the newer generation immunosuppressants, whose use is suggested to positively influence

axonal regeneration, may increase the difference in the regeneration achieved by immunosuppressed and non-immunosuppressed models, thus providing a more testing environment for the Sandwich graft.

Finally, since this project was completed the technology now exists to label Schwann cells so that their position and origin can be tracked. The ability to distinguish between host and donor Schwann cells directly is a major advance. Further work comparing the empirical, clinically achievable Sandwich graft with other conduit materials containing nerve segments and cultured autologous and allogenic Schwann cells would provide further evidence on Schwann cell function and antigenicity while allowing more opportunities to test the regeneration potential of allogenic nerve tissue.

While the unethical label attached to peripheral nerve allografting is difficult to shift, the recent advent of limb allotransplantation indicates that investigation of peripheral nerve allografting is still required if this surgical advance is to achieve any greater potential than just the technical feat.

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APPENDICES

Appendix 1 - Solutions

Zamboni's Solution

Solutions are mixed in the following proportions:

85ml 2% paraformaldehyde in 0.1% phosphate buffer (see below)
15ml saturated picric acid

For 2% paraformaldehyde, 20g paraformaldehyde is dissolved in 800ml 0.1% phosphate buffer with heat (max. 60°C), stirring until solution is clear. The volume is then adjusted to 1 litre and left to cool.

Saturated picric acid is added to make Zamboni's solution which is stored at 4°C until use.

0.01M Phosphate Buffered Saline (PBS)

Makes 10 litres

The following are added to 5 litres of distilled water, stirring until dissolved:

NaCl	87.9g
KH_2PO_4	2.72g
$Na_2HPO_4(H_2O)_{12}$	23.9g
Or Na_2HPO_4	11.35g

The resulting solution is then left for a few hours before adding the remaining 5 litres of distilled water. After standing for at least 8 hours, the pH is checked and adjusted to pH 7.3 (NaOH/HCl).

PBS – Sucrose

15% sucrose + 0.1% sodium azide in PBS (as above)

For 1 litre, dissolve the following in 700ml PBS:

Sucrose	150g
Sodium azide	1g

When dissolved, make up to 1 litre with further PBS.

0.1M Phosphate Buffer

Solution A	31.2g $NaH_2PO_4.2H_2O$ dissolved in 800ml distilled water and made up to 1 litre
Solution B	28.4g Na_2HPO_4 dissolved in 800ml distilled water and made up to 1 litre

Approximately 23ml of Solution A are added to 77ml of Solution B until the pH is 7.2-7.4. The pH can be adjusted by adding small volumes of either solution as required. The solution is then diluted 1:1 with distilled water to give a 0.1M solution.

2.5% Glutaraldehyde

25% electron microscopy grade glutaraldehyde is diluted with 0.1M phosphate buffer in a ratio of 1:9.

PBS + 0.2% Triton X

Mix 2ml of 0.2% Triton X-100 with 1 litre PBS

Glycerol/PBS mountant

1g of 1,4-diaza bicyclo[2.2.2]octane is added to 4ml PBS and then mixed with 35ml glycerine.

0.05% Hydrogen Peroxide

Mix 0.175ml of hydrogen peroxide (AnalaR™, BDH Laboratory Supplies, U.K.) with 99.825ml of distilled water, stir thoroughly.

0.1M Sodium Acetate buffer

82.03g of sodium acetate is dissolved in 700ml of distilled water. The solution is made up to 1 litre then the pH is adjusted to 6 using NaOH or acetic acid.

Antibody Diluent

For 100ml solution. Dissolve the following in 100ml of PBS:

Bovine serum albumin (BSA)	0.1g
Sodium azide	0.1g
Triton X-100	30ml

Appendix 2 – Glass microscopy slide preparation

Vectabond Coated Slides

Glass microscopy slides were treated in the following manner to enhance adhesion of the longitudinal tissue sections utilised here and prevent loss during the multiple steps and rinses involved in the staining techniques employed.

1. Slides were cleaned in dilute chromic acid and thoroughly rinsed in water.
2. Slides were immersed in acetone for 5 minutes, removed and thoroughly drained.
3. VECTABOND™ reagent was prepared by mixing 7ml with 350ml of acetone.
4. Slides were then placed in VECTABOND™ reagent treatment solution for 5 minutes.
5. Finally slides were dried thoroughly at 37°C.

Appendix 3 – Semi-thin section staining

Acridine orange and thionin staining of semi-thin sections

Stain preparation:

Thionin

0.05g thionin is dissolved in 22.5ml absolute alcohol. 22.5ml of distilled water is added plus 5ml of 0.1M sodium hydroxide, and then filtered.
Fresh stain should be prepared for each staining run.

Acridine orange

Prepare in a fume cupboard
Add 0.25g acridine orange to 20ml distilled water and 5ml 0.1M sodium hydroxide solution and heat gently to dissolve. Filter before use.

Staining method:

Dry glass mounted 1μm semi-thin sections on a hot plate.
Flame the underside of the glass slide through a Bunsen burner flame to fix the section to the slide.
Stain with thionin for 45-60 seconds at 65-70°C on the hot plate.
Wash well with distilled water.
Stain with acridine orange for 30 seconds at 65-70°C on the hot plate.
Wash well with distilled water and dry on the hot plate.
Mount in DPX mountant

Appendix 4 – Presentations to Learned Societies

British Association of Plastic Surgeons - Summer Meeting 2001

Sandwich Grafting: An Assessment of the Long-term Effects of this technique on Regeneration through Peripheral Nerve Allografts

F.J. Hogg, C.J. Green, G. Terenghi.

British Association of Plastic Surgeons - Summer Meeting 1998

Sandwich Grafting: Can host Schwann cells improve regeneration through peripheral nerve allografts?

F.J. Hogg, C.J. Green, G. Terenghi.

British Association of Plastic Surgeons - Winter Meeting 1996

Is immunosuppression for peripheral nerve allografts necessary?

F.J. Hogg, G. Terenghi, C.J. Green.