

From the Department of Clinical Science and Education
Södersjukhuset, Karolinska Institutet,
Stockholm, Sweden

Central and peripheral adaptation after nerve repair with a synthetic biocompatible adhesive

Thomas Landegren



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*To Lotta, Märta, Max och Olle
..... och lilla mamma förstås*

ABSTRACT

The epineural repair technique is currently the accepted standard for peripheral nerve repair. However, due to the demanding nature of this method, it is not uncommon to delay or even omit action, especially in situations which are associated with extensive traumatic injuries. Some of these situations may involve natural disasters, (traffic) accidents, or times of war. Consequently, there is a need for a complementary surgical technique that offers a rapid and reliable primary repair of transected peripheral nerves. Suggestions for techniques using a biocompatible adhesive have been proposed to meet the need. An adhesive would need to have certain qualities such as being simple to apply, having good binding strength with the ability to adhere in a moist environment and being biodegradable in nature and compatible with tissue. In this research, our aim was to create an experimental model that evaluates peripheral nerve transection and repair with a synthetic adhesive and thereafter to compare the results with conventional microsuturing. We also compared two different synthetic adhesives with regard to the cytotoxic effect of a human neuroblastoma cell line.

In *Studies I, II, and IV* unilateral sciatic nerve transections were performed on rats. The nerve endings were readapted microsurgically with cyanoacrylate or epineural sutures. Local tissue showed an increased accumulation of ED-1-immunoreactive macrophages on both sides of the repair site. Neurofilament labelling was less pronounced distal to the repair site seven days after reparation with cyanoacrylate compared with sutures. After six months, when reinnervation had been completed, we examined the tibial branch to the lateral gastrocnemius muscle and the caudal sural cutaneous nerve, in both cases electrophysiologically, as well as morphologically. Functional reinnervation of motor and sensory nerves was observed in both groups. This was shown by equivalent recovery of motor and sensory conduction velocities, as well as motor nerve action potentials. Histological examination showed no significant difference with regard to the mean diameter, fibre density, or the number of regenerated myelinated motor and sensory axons distal to the repair site. The difference in ED-1-immunoreactivity on each side of the repair site was less noticeable. Using the cholera toxin B technique of retrograde axonal tracing over the repair seam, the morphology, the number and the three-dimensional location of α -motoneurons innervating the lateral gastrocnemius muscle were evaluated and related to the recorded wet muscle weight. Regardless of which repair method was used, the redistribution of the α -motoneuron pool had increased, was disorganised, and was scattered throughout a larger volume of the spinal cord grey matter. The synaptic coverage had decreased and the muscle weight was reduced.

In *Study III* the cytotoxic effect of ethyl-cyanoacrylate was examined on a human neuroblastoma cell line (SH-SY5Y) and compared with the effects of butyl-cyanoacrylate (Histoacryl®), commonly used for skin closure. Both were applied to confluent SH-SY5Y cultures. The cultures were photographed and analysed digitally. At corresponding intervals, cell death was quantified using a ^{51}Cr release assay. In cultures exposed to either of the two adhesives, cell death was observed predominantly

in conjunction with the adhesive causing a halo devoid of cells, which diminished over time. At the 28-day mark, cells had reached the margin of the adhesive in the ethyl-cyanoacrylate group. The surviving cells showed neurodegenerative properties up to three days post exposure. The cell death, indicated by the ^{51}Cr assay, rapidly decreased during the first 14 days. No significant differences were found between the adhesives.

Conclusions: Anastomosis of a transected peripheral nerve with ethyl-cyanoacrylate adhesive supports morphological and functional recovery, a recovery which is comparable to that of conventional epineural sutures. Ethyl-cyanoacrylate causes a transient cytotoxicity, which appears to induce an increased local inflammatory reaction, leading the way to accelerated Wallerian degeneration.. Ethyl-cyanoacrylate does not have any negative influence on the selectivity of motor reinnervation following nerve transection and repair compared to that following conventional microsuturing. This method could therefore offer benefits over conventional sutures in the reconstruction of peripheral nerves.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in text by their Roman numerals:

- I. **Landegren T**, Risling M, Brage A, Persson JK.
Long-term results of peripheral nerve repair: a comparison of nerve anastomosis with ethyl-cyanoacrylate and epineural sutures.
Scand J Plast Reconstr Surg Hand Surg. 2006;40(2):65-72

- II. **Landegren T**, Risling M, Persson JK.
Local tissue reactions after nerve repair with ethyl-cyanoacrylate compared with epineural sutures.
Scand J Plast Reconstr Surg Hand Surg. 2007;41(5):217-27

- III. **Landegren T**, Risling M, Persson JK, Sondén A.
Cyanoacrylate in nerve repair - transient cytotoxic effect.
In press

- IV. **Landegren T**, Risling M, Persson JK.
Selectivity of reinnervation of the gastrocnemius muscles after nerve repair with ethyl-cyanoacrylate.
Manuscript

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LIST OF ABBREVIATIONS

| | |
|------------------|--|
| α -MNs | α -motoneurons |
| BDNF | Brain-derived neurotrophic factor |
| BNB | Blood-nerve barrier |
| ⁵¹ Cr | Chromium - 51 |
| CA | Cyanoacrylate |
| CGRP | Calcitonin gene-related peptide |
| CMAP | Evoked compound motor action potential |
| CNS | Central nervous system |
| CTB | Cholera toxin subunit B |
| Cy2 | Conjugated antibody with fluorescent dye (green) |
| Cy3 | Conjugated antibody with fluorescent dye (red) |
| DRG | Dorsal root ganglion |
| ECA | Ethyl-cyanoacrylate |
| ED-1 | Antigen expressed by macrophages in rat. |
| EMG | Electromyography |
| GAP-43 | Growth associated protein-43 |
| Glut-1 | Glucose transporter 1 |
| HRP | Horseradish peroxidase |
| IR | Immunoreactivity |
| MCV | Motor conduction velocity |
| NGF | Nerve growth factor |
| PBS | Phosphate buffered saline |
| PNS | Peripheral nervous system |
| RLM | Retrogradely labeled motoneurons |
| SCV | Sensory conduction velocity |
| SNAP | Sensory nerve action potential |

THESIS AT A GLANCE

Paper I

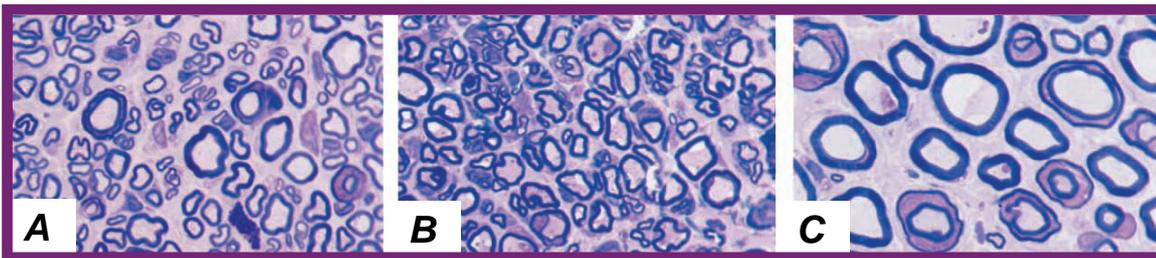
Can a transected peripheral nerve heal after repair with a synthetic adhesive?

Methods: Peripheral nerve injuries in rats were experimentally repaired with either microsutures or ethylcyanoacrylate

Evaluation times: Six months

Evaluation methods: Electrophysiological and quantitative histological examinations

Conclusions: Anastomosis of the nerve with (ECA) supports morphological and functional recovery, comparable to that of conventional epineurial sutures



Regenerated motor nerve fibres after ECA repair (A), epineurial sutures (B) and normal controls (C).

Paper II

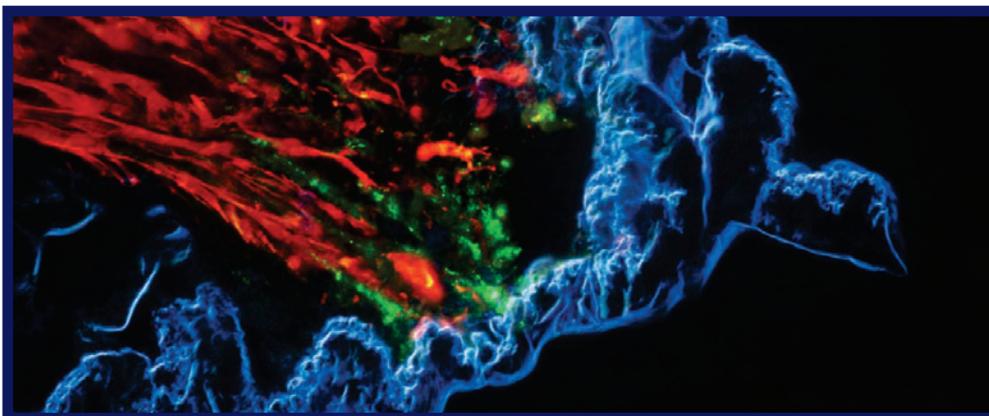
Is the local tissue reaction around the seam different after peripheral nerve repair with ECA to conventional techniques?

Methods: Macrophages and nerve fibres adjacent to the repaired site were examined after peripheral nerve repair

Evaluation times: Seven days and six month

Evaluation methods: Immunohistochemistry and laser scanning confocal microscopy.

Conclusions: ECA induces an increased inflammatory reaction, which may accelerate Wallerian degeneration, and could therefore be beneficial over conventional sutures for reconstruction of peripheral nerves.



Red = nerve fibres, Green = "Path finding" regenerating nerve fibres, Blue = ECA

Paper III

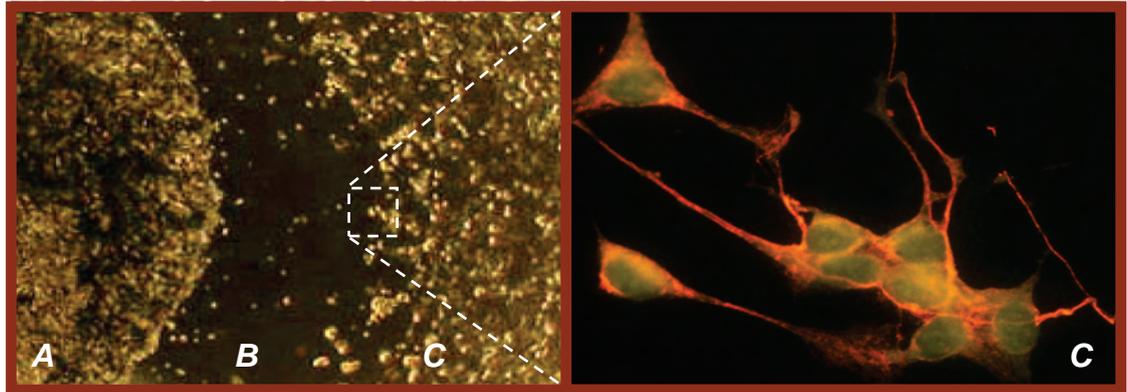
Ethylcyanoacrylate, what about the cytotoxicity?

Methods: Neuronal cells - exposed to ECA or Histoacryl® - a skin closure adhesive.

Evaluation times: 24 hours, 7, 14, 21 and 28 days

Evaluation methods: Cell morphology and detection of cell death using a ⁵¹Cr assay

Conclusions: There are cytotoxic effects with both these adhesives. The transient nature of the toxicity may explain the promising results using ECA for nerve repair.



ECA (A), Halo devoid of cells (B) and Neuronal cells (C).

Paper IV

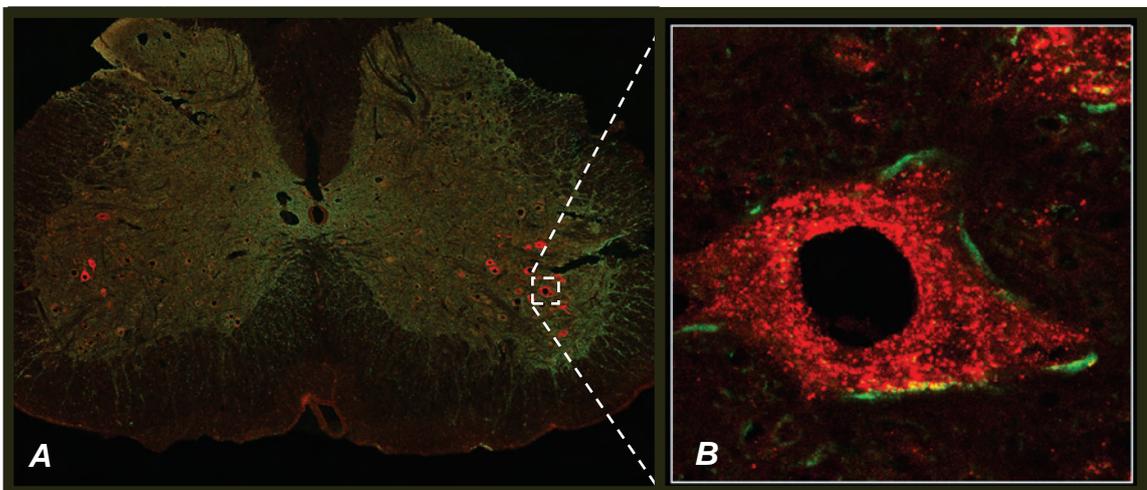
Does ECA affect the selectivity of muscle reinnervation?

Methods: Retrograde axonal tracing with Cholera toxin B technique were used to evaluate α -motoneurons innervating the lateral gastrocnemius muscle after sciatic nerve repair with either ECA or epineural sutures

Evaluation times: Six month

Evaluation methods: Morphological examinations with fluorescence or confocal microscopy

Conclusions: The selectivity of motor reinnervation following peripheral nerve transection, and repair with ECA, is comparable to that of conventional microsuturing.



L5 spinal cord after nerve repair with ECA (A). α -motoneurons (B) with synaptic terminals (Green)

INTRODUCTION

In Sweden, the incidence of transectional nerve injuries reaches approximately 1.5/10,000 citizens each year. This number varies geographically, depending on the prevalence of heavy industry and mechanised agriculture (1). Injuries to peripheral nerves are especially common in the upper extremities and are predominantly caused by direct mechanical trauma, resulting in transection, crush injury, traction, or avulsion (2-3). The severity of each case varies from minor injury, such as digital nerve damage, to cases of a severe nature, such as brachial plexus injury. Some injuries affect one of the main nerve trunks. In these cases, the resulting injury will probably be a long-lasting disability (4) due to pain, loss of sensation, and weakening of muscles, as well as a reduced range of motion. The clinical outcome following nerve injury is often unsatisfactory, and normal functionality will not be regained even if the nerve has been repaired (5). Consequently, a substantial number of patients will have reduced ability to function in their workplace. Furthermore, their functionality involving other aspects of daily activities will be significantly compromised as well. While the inability to use one's hand to touch, to explore, and grip in a normal way is a considerable disability for the patient; the economic consequence caused by a patient's inability to function adequately is a great financial concern for society. According to Rosberg et al. (6), the total incurred cost to society after a median nerve injury is €51,238.00 for one employee. The corresponding amount for an ulnar nerve injury is €31,186.00. These costs are largely related to the loss of efficiency and productiveness, as well as instatements of new training programmes for the victim. One can conclude that patients who are victims of peripheral nerve transection injuries are often employed (mean age of patients, 30 years) (6).

The above indicates that peripheral nerve injury is a serious health concern to society and that there is a strong incentive to improve the functional outcome after peripheral nerve repair. The epineural repair technique is currently accepted as the gold standard of peripheral nerve repair. However, this procedure is technically demanding, it is time-consuming, and it requires substantial material resources. These are factors which frequently seem to cause delayed action or even a lack thereof. This is particularly prevalent in situations associated with extensive traumatic injuries as is often the case when natural disasters strike, in large-scale (traffic) accidents, or during times of war. Consequently, there is a need for a complementary surgical technique which can provide a rapid and reliable primary repair of transected peripheral nerves. In order to meet this demand, the aim of which is to improve the functional outcome of nerve injuries, several bonding techniques have been proposed as options to sutures for achieving proper coaptation of the nerves. This thesis describes a new, yet simple, approach to repairing peripheral nerves which have been transected. With this method, a single synthetic adhesive is used to reconnect two nerve endings which have been transversely divided. This method will be of clinical value because of its relatively inexpensive, yet readily available, nature. Hence, it is a potential alternative to microsutures in the treatment of traumatic peripheral nerve injuries.

Historical background

During the Hippocratic era, physicians and scientists alike did not yet understand the anatomical difference between a nerve and a tendon. However, Hippocrates (460–370 BC) himself inferred certain conclusions about peripheral nerves and their injuries. In his work *Aphorisms*, he introduced a concept in which he explained that a nerve is not able to be restored, or reconnect itself, once it is accidentally transected. With these statements he made it clear that nerve damage is considered to be untreatable (7). Moreover, physicians of his School warned that an injury to a peripheral nerve may cause spasm and may result in a cruel death for the victim. The proclamation in which Hippocrates stated that a divided nerve neither grows nor can reunite itself deterred physicians from attempting nerve surgery for many centuries (8). During the same period, another leading scientist, Aristotle (384–322 BC), claimed that the heart is the origin of the nerves. He also claimed that there is no connection between the brain and the sense organs (7). With regard to repairing severed nerves, history remained silent until Galen of Pergamon (131–201 A.D). Galen was a surgeon to the Roman gladiators, and the first physician to distinguish between motor and sensory nerves. He reported incredible results where severed nerves had been sutured. However, these miracles were never accomplished by Galen himself (9). Mainly due to cultural and religious prejudice against exploring the secrets of nature, little progress in science was made during the first 1500 years A.D. In the beginning of the sixteenth century, Paracelsus, Servetus, and Vesalius, among others, began to criticise the obligation to use old philosophical works, like those of Aristotle, as the only acceptable source of medical information. It was their opinion that this type of thinking would impede human progress. The results of their opinions and accomplishments were mostly fatal to themselves but led to the beginning of actual scientific activity (10).

The modern concept of surgery on peripheral nerves started essentially with Gabriele Ferrara's work (1543–1627) in Italy. He was the first to give a precise description of suturing of the stumps of a transected nerve. In his treatise, Ferrara gave a detailed portrayal of the equipment which he used in peripheral nerve surgery. He stressed the importance of identifying the nerve stumps in the wound and, accordingly, he described a technique for nerve-end dissection from surrounding tissues. Today, almost half a millennium later, we are still practicing the same *modus operandi* introduced by this prominent author (11). Although the founder of modern medicine, Hippocrates, revealed a singular approach to the treatment of peripheral nerve injuries, Fernandez quotes a well-known surgical treatise from the 16th century by the Turkish surgeon Ibrahim: "...overnight, during a travel in a caravan along the Tigris river, Hippocrates (460–377 BC) probably performed the first nerve repair of one interrupted nerve using for suture the hair of a woman. The patient was a thief who received a cut injury at the level of one ankle..." (12). History or legend? The truth of stories related to the father of medicine will probably never be determined, nor is it likely that the time of the first peripheral nerve repair will ever be established. Regardless of the true historic development, one may conclude that the scientific principles which supported the decision-making with regard to nerve repair appear to have been based on random guesswork and arbitrary facts rather than true science. As for the nerve cell and the

nerve fibre, the generally accepted view until the mid-nineteenth century was that they were considered to be independent elements.

A milestone in the understanding of nerve injury was reached in 1850 by Augustus Waller, who challenged the prior views when he clearly described the phenomenon of “Wallerian” degeneration and the loss of the distal nerve elements and new fibres regenerating from the proximal stump after axonal lesion (13). His proclamations, were, however, not generally accepted until Santiago Ramón y Cajal (1852–1934, Nobel Prize, 1906) proved the neuron theory, which explains that the nervous system is made up of independent cells, and also identified the growth cone as the apparatus that directs axonal growth during both the developing and regenerating stages (14). These developments dramatically turned attention to the study of nerve repair. Further understanding and an increased knowledge of the anatomy, function, and physiology of the nerve have resulted in a more precise understanding of the nerve healing process. This, in turn, has initiated the establishment of rational strategies for nerve repair. One has gone from wild speculation to a more predictable reality.

Surgical developments continued in the twentieth century. Advancements in more sophisticated surgical equipment along with the use of microsurgical atraumatic techniques have allowed more technically precise methods and reasonably good fascicular alignment, and improved functional outcomes as well. Interestingly, surgeons have not recently been able to further refine the techniques. Instead, the interest has been focused on the importance of microenvironmental factors regulating nerve regeneration. Throughout the past decades, more and more knowledge has been acquired about both the cellular and molecular mechanisms of the regeneration process (5). Thus, various methods have been tried in order to improve regeneration in experimental models. Dating back to early history, there are documented and descriptive narratives regarding the bonding of biological tissue. Paul of Aegina in 600 A.D. combined agglutination and sutures to reconnect severed nerve endings. Approximately 600 years later, Roger of Parma used egg albumin as an adhesive during efforts to repair damaged nerves.

The clinical treatment has not developed accordingly, however. Sutures are still the most frequently used method in cases of traumatic nerve injury. In other words, anatomic axon-to-axon reconnection with microsutures remains to be the golden surgical standard.

The peripheral nervous system

The nervous system is divided into two parts: one central and one peripheral. The central nervous system (CNS) comprises the brain and spinal cord. The peripheral nervous system (PNS) includes the cranial nerves, the spinal nerves with their roots, and the peripheral nerves, as well as the peripheral components of the autonomic nervous system (ANS). Primary sensory neurons are located in the dorsal root ganglia (DRG) of the PNS; ganglion is defined as a collection of nerve cell bodies in the PNS, observable as a swelling of the spinal nerve root. The sensory neurons of the somatic nervous system project branches both centrally to the CNS and peripherally through the dorsal roots to peripheral targets. Sensory neurons are activated by sensory input

(vision, touch, hearing, etc.) and lead afferent information from the inside of the body and the environment to the CNS (15). The size of the sensory cell body correlates with the size of the axon and thus with the conduction velocity. Large neurons transmit sensation and proprioception while small neurons deal with pain and temperature. Spinal α -motoneurons extending axons into the PNS have their somata located in the ventral horn of the spinal cord and their axons leave the CNS through the ventral roots carrying efferent signals to the effector organs (muscles). Recovery of motor function following nerve injury and repair is usually superior to that of sensory function in adults. The dorsal and ventral roots are attached to the spinal cord and the attachment site is regarded as the border between the PNS and the CNS (16).

In the limbs the peripheral nerves consist of bundles of nerve fibres, with a variable mixture of motor, sensory, and autonomic fibres. Distally, these nerves become increasingly segregated into more functionally specific fascicles. The nerve fascicles are separated by the surrounding perineurium, while the nerve trunks are surrounded by the epineurium. The perineurium is a strong multilaminated connective tissue sheath composed of flattened cells with a basement membrane on both inner and outer aspects. It protects the contents of the endoneurial space, acting as a mechanical barrier to external trauma, and constitutes a diffusion barrier as well. Inside the fascicle, a delicate packing of loose connective tissue called endoneurium, consisting of basal laminae, capillaries, collagen fibres, endoneurial fibroblasts, and macrophages, surrounds the individual nerve fibres with their associated Schwann cells (17-18).

The functional unit of the peripheral nerve is the nerve fibre. Nerve fibres can be classified as myelinated or unmyelinated. Both types of fibres are associated with a longitudinally arranged chain of Schwann cells, but the organisation between the axon and the Schwann cells differs in the two types of fibres. In the myelinated fibre, only one axon is associated with the chain of Schwann cells. Each Schwann cell plasma membrane is wrapped in several layers around the axon, creating an insulating myelin sheath. This axon-Schwann cell unit is surrounded by a basal lamina tube. The sheath is not continuous over the entire length of the axon but is interrupted at different intervals. These non-myelinated gaps between two adjacent Schwann cells are termed nodes of Ranvier. The electric impulse travels along a myelinated axon by “jumping/skipping from one node to another”, and this saltatory propagation of action potentials increases the conduction velocity, which can reach over 100 m/s in the largest axons (19). In the non-myelinated nerve fibre, several axons are associated with each chain of Schwann cells. These axons are thin, and there is no gap between adjacent Schwann cells, which leads to a slow conduction velocity (18).

The peripheral nerve is a well vascularised structure receiving its vascular supply from an extrinsic system of vessels approaching the nerve trunk at various levels along its course, and an intrinsic system, which consists of intercommunicating vascular plexuses in all the different layers (20-21). The extreme length of the neuron puts special demands on it. Through axonal transport, there is a constant flow in a distal direction of essential substances such as transmitters, enzymes, and cytoskeletal components. There is also a similar proximally directed transport of neurotrophic substances necessary for the normal function of the nerve cell body (21-22).

Peripheral nerve injury and regeneration

Classification of nerve injuries

When discussing nerve lesion and repair it is necessary to distinguish between different degrees of nerve injury. The two most widely used classification systems, according to symptoms, pathology, and prognosis for peripheral nerve injury, are those of Seddon (23) and Sunderland (24). This thesis deals with the most serious degree of injury: Transection of the entire nerve trunk (the fifth degree of nerve injury) according to Sunderland or *neurotmesis*: "... a nerve that has been completely divided. The injury produces a lesion which is in every sense complete." This refers to the Seddon classification.

The distal segment

After complete axonal transection, the neuron undergoes a number of degenerative processes, followed by attempts to regenerate. Subsequently, when the peripheral nerve axon is disconnected from the cell body, its distal segment gradually degenerates and eventually disappears, collectively referred to as Wallerian degeneration (25). This series of cellular events begins within hours of injury and is completed within 6–8 weeks. In the axon, several changes occur, including leakage of intra-axonal fluid and swelling of the distal nerve segment. Macrophages invade the site of injury and, in a co-ordinated effort together with Schwann cells, start to clear the injured area of debris (26). Schwann cells, which are devoid of contact with axons, transiently proliferate and differentiate into a phenotype that aligns longitudinally in typical cell columns within tubes of remaining endoneurial basal lamina, forming so-called bands of Büngner (27). Each Schwann cell column performs the function of a conduit which provides the pathway and scaffolding of the regenerating axon as far as the original target organs (28). This Schwann cell column thus provides the regenerating axons with an environment favourable for growth and is the source of a various kinds of trophic factors promoting axonal growth (18, 29). However, endoneurial tubes or Schwann cell columns that do not receive any regenerating axons will lose their capacity to support regeneration, shrink, and eventually become obliterated by scar tissue (30).

The nerve cell body and the proximal segment

As a reaction to a peripheral axotomy, the nerve cell body of surviving neurons displays several morphological and metabolic changes. Within 2 to 3 days of the injury, classical signs consisting of chromatolytic changes, nuclear eccentricity, and cell swelling are consistent features of the reaction in neurons whose axons are shielded from the PNS (changes that may reverse as recovery occurs) (31). These changes, referred to as the cell body reaction (31), reflect a modification in the metabolic priority from a transmitting mode to a growth mode, i.e. involving alterations in the expression of a multiplicity of genes and growth-associated proteins, such as GAP-43, all of which have the potential to promote axonal regeneration (32). The closer the injury is to the

cell body, the more severe the cell body reaction and, finally, the outcome will be (33). Axonal lesion, which disrupts the contact between the cell body of the nerve and the peripheral target organ, leads to death in up to 35% of the sensory neurons (34). However, a common opinion is that motoneurons are much less susceptible to die compared to sensory neurons after peripheral nerve injury (35).

In the proximal stump, retrograde Wallerian degeneration usually involves only a short segment of the axon (a few internodes). As soon as a few hours after axotomy, the injured axons give rise to several neuronal sprouts emerging from nodes of Ranvier (36). The terminal tip of the regrowing axons, or growth cone, responds to contact guidance cues, exploring the environment and searching actively for a suitable matrix and milieu to support the axonal growth; for a review, see Ide (18). Schwann cells from the distal stump are the most effective substrate for producing and directing regeneration, and a reunion of the nerve endings is therefore a necessary prelude for the sprouting axons, emerging from the proximal stump to pass through a successful regeneration (37). The interaction between Schwann cell membrane and growth cone is mediated by cell adhesion molecules (CAMs) expressed by both elements (Rutishauser, 1993). These adhesion molecules have been suggested to be upregulated by growth factors produced by Schwann cells and to thereby potentiate axonal regeneration (38).

Following the repair of a transected nerve, regenerating axons have to bridge over the gap between the proximal and distal nerve stumps. During the early stages of regeneration, this gap is filled with fluid, containing different neurotrophic factors, at which point a longitudinal fibrin matrix is formed between the nerve endings. This fibrin matrix also contains adhesive molecules like fibronectin and macrophages, as well as other inflammatory cells. Subsequently, it is invaded by fibroblasts and developing blood vessels. Schwann cells migrate from both the proximal and distal segments in order to sustain the outgrowing axons emerging from the proximal segment and to conduct to the distal stump. In a mesothelial chamber axons have been shown to be able to traverse a 10-mm gap (39). However, far from all regenerating axons will reach the distal stump. Success depends on multiple factors, including the severity of the original injury, the extent of the scar formation, and the delay before the axons reach the distal stump.

Axons that successfully enter the endoneurial tubes in the distal stump stand a good chance of reaching the end organ. The regeneration of an axon, however, is not synonymous with regaining functionality. The rate of axonal regeneration depends on several factors. Regeneration is better in a proximal target organ and among younger individuals (40). Clinically, an estimate of 1 mm/day is generally applied. Even after the axon has reached its target, a maturation process has to develop, including remyelination, axonal enlargement, and the establishment of connections with the end organ, before functional recovery may come about. A nerve injury also results in changes in the effector organs, and the state of these by the time of reinnervation will affect the result. After a moderate to severe injury, an incomplete motor recovery may result from numerous factors. Muscle fibres atrophy quite rapidly (40). A few weeks after an injury, fibrotic changes may be detected in the muscle. Intramuscular fibrosis may limit contractile efficiency, and aberrant regeneration may reduce the synergy of

contraction (41). Even with successful reinnervation, the muscle will rarely regain its normal strength. In cases where significant motor recovery occurs, the functional outcome may be impaired by concomitant sensory deficits, particularly in proprioception. Repair after more severe nerve injuries will never result in complete recovery of the senses. This is probably related to a combination of factors, including failure of sensory axons to reach the skin, cross-reinnervation, and possibly degeneration of sensory receptors (40). Furthermore, axonal misdirections or path-finding errors have been shown to be particularly problematic with proximal injuries to large mixed nerves (37).

Management of peripheral nerve injury

In an ideal situation, a primary and tension-free repair with a proper fascicular alignment is always preferred when a peripheral nerve is injured by sharp transection. A prerequisite for this is a clean and well-vascularised wound with no or minimal damage to the nerve endings, and a precondition for adequate soft tissue coverage. Primary nerve repair is the term used when a peripheral nerve is repaired within the first week after the injury occurred (42). It is generally considered a *delayed primary repair* when performed after the first 3 to 4 days of the injury. In terms of timing, nerve repairs performed more than a week after an injury are secondary repairs. However, experimental (43-44) as well as clinical (45-47) studies have shown that the regenerative capacity after nerve lesion is improved if the interval between injury and repair is short.

In current surgical practice the continuity of the nerve trunk is restored by microsurgical end-to-end nerve repair, either by single sutures passed through the epineural sheath or by fascicular repair when groups of corresponding fascicles are approximated with microsutures. Traditionally, epineural repair is considered to be most beneficial for sharp injuries without loss of nerve substance, whereas fascicular repair has been considered more suitable for cases where trimming of the nerve ends is necessary. However, while these statements hold true, current trends show that fascicular repair is no longer used on a wide basis (48). Experimental studies have shown that although this technique ensures a more precise fascicle alignment of regenerating axons, there is little evidence that it is superior to the less exact, but simpler, epineural repair on comparing functional outcomes (49-50).

After complete nerve transection, the inherent elasticity and the tensile strength of the nerve causes a retraction of the proximal and distal nerve endings, thereby forming a gap between the two sites (51). When nerve repair is performed acutely, the gap is roughly equivalent to the nerve diameter of the intact nerve, making primary end-to-end repair possible. As soon as three weeks after injury, the retraction of the nerve stumps may reach an intervening gap, which may make it impossible to reconnect the nerve endings without considerable tension (48). The limiting factor is not the mechanical tension as such, but vascular aspects (52). Even a slight tension over the repair site compromises the intraneural microcirculation to the nerve endings (53) and when end-to-end nerve repair is considered, the functional outcome is affected adversely if it is not possible to readapt the nerve endings without considerable tension

(54). An experimental study in a rat sciatic nerve ischemia model has shown that the blood flow is inversely proportional to nerve tension (52). A wide variety of ranges regarding safe limits have been reported in both experimental and clinical studies. Lundborg and Rydevik observed a decrease in the blood flow with only 5% elongation, and complete cessation of blood flow after approximately 15% elongation of the nerve (55). These results have led to an established limit of elongation in unscarred peripheral nerve of 8–10%, or the point at which blood flow has been shown to decrease by 50%. Moreover, excessive tension over the repair site can lead to rupture of the repair seam or an inadequate coaptation of the fascicles (56).

In daily clinical practice, when the surgeon is confronted with the task of achieving a successful peripheral nerve repair, it is often a considerable challenge to accurately determine the limits of tension using nerve elongation. The tension required to bring the proximal and distal nerve ends together must be assessed, usually accomplished by grasping the two cut ends with forceps and bringing them together. If the tension seems to be excessive, the surgeon is faced with three choices: to mobilise the nerve endings proximally and distally in an attempt to achieve more length and reduce tension in the nerve segments, or to use an alternative technique such as nerve graft, or an end to-end/end to-side nerve transfer. The dissection necessary to achieve mobilisation may itself be problematic since it has been shown to compromise blood supply and thereby bring about scar formation which blocks axonal regeneration and ultimately results in neuroma formation (57). On the other hand, autologous nerve grafting, which is still the preferred technique for bridging nerve gaps (54), is accompanied by several disadvantages. Sacrificing a healthy nerve for grafting will always cause patient donor site morbidity, such as additional scarring and loss of sensation, and, occasionally, a painful neuroma or hyperaesthesia may occur at the harvest site (58). The sural nerve is the most frequently used graft material in the reconstruction of larger nerve trunks. This nerve has the advantage of being able to bridge large gaps and can be excised to a length of 30 to 40 cm from each leg. Other nerves, e.g. the medial and lateral cutaneous nerves of the forearm or the lateral femoral cutaneous nerve, are occasionally used as well. Furthermore, nerve grafting that forces the regenerating fibres to cross over two repair sites increases the risk of axonal loss due to scar formation and misdirection into the surrounding connective tissue at each suture line (59-60) and, despite advances in microsurgical techniques, the functional outcome after a nerve grafting procedure is often unsatisfactory (45). Technical details of nerve grafts, nerve conduits, nerve transfers, or end to-side nerve repair will not, however, be outlined here. Although the traditional technique of neurorrhaphy lacks tension, it has been shown, by means of experiments directing nerve repair under modest tension, to actually do better than a tension-free nerve graft over the same regenerating distance (61). Accordingly, in spite of the available microsurgical techniques, posttraumatic nerve repair continues to be a major challenge in reconstructive surgery (62).

Even though microsurgical suturing continues to be the standard technique for repairing transected peripheral nerves, it necessitates surgical excellence, requires substantial material resources, i.e. microsurgical precision instruments, adequate magnifying equipment, etc. (63) and may be difficult to apply when surgical access is

limited. Those are factors which not infrequently seem to cause delayed action or a lack thereof, especially in situations large numbers of extensive traumatic injuries, such as in natural disasters, large-scale (traffic) accidents, or in wartime. Furthermore, this surgical procedure will always carry the risk of injuring the tissue and jeopardising the blood supply to the fascicles (64-65), and it will not result in perfect coaptation of the internal fascicular structures, which is known to hamper the growth of regenerating axons and, ultimately, the recovery of the nerve function (66).

When considering the drawbacks of sutures, as well as the pitfalls of secondary nerve reconstruction by bridging gaps to avoid unfavourable tension, one may conclude that there is a need for complementary surgical techniques which will provide rapid and reliable primary repair of transected nerves. Several adhesive techniques have been proposed as options for sutures to achieve proper coaptation of nerves. Such adhesives should be simple to apply, have good binding strength, be able to stick in a moist environment, and be biodegradable and compatible with living tissue. A variety of different biological or artificial bonding materials have been used experimentally with variable results. The advantages of the synthetic adhesive cyanoacrylate as a tissue adhesive for clinical use have been described in a variety of medical fields (see below). The possibility that cyanoacrylate could be a method of choice as a nerve repair adhesive is considered in this thesis.

Tissue adhesives

Historically, the one option for closure of soft tissue has basically been limited to suturing (needle and thread). Traditional suturing enables meticulous tissue adaptation, but sutures may induce tissue reactivity, and superficially located sutures not infrequently require removal. Tissue adhesives as an alternative in clinical practice have recently made progress. The concept of adhering, rather than suturing or stapling, planes of tissue is attractive since it is fast-acting, assures complete closure, and is without risks of needle stick injuries to the surgeon and his/her assistant. The adhesive is applied over the surgical areas and holds the edges or surfaces together until healing has occurred. Numerous technologies have been tried with some successful and some doubtful, or outright failing, results. Limitations according to the sealing procedure occur in a number of areas: administration, bonding strength, toxicity, degradation, and safety. Over the last few decades, however, advances have been made in a wide range of technologies and the introduction of tissue adhesives has been received enthusiastically as they may result in equivalent tensile strength, improved appearance of the scar, and a lower infection rate compared to sutures or staples (67). Foremost and most successful among these tissue adhesives are cyanoacrylates and fibrin-based sealants. Another promising adhesive technology is laser welding, a process in which components in the extracellular matrix of the connective tissues integrate with the repair site when laser energy is applied.

The evolution of microsurgery has enabled the surgeon to perform microcoaptation of nerves with technical expertise, which, historically, has been impossible. However, when a peripheral nerve laceration is repaired with microsurgical stitches, the internal fascicular structure at the suture line becomes disorganised.

Misdirection of the regenerating axons is considered one of the frequent factors hampering the outcome/recovery after nerve repair (66). Consequently, there is a need for new methods for the handling of peripheral nerve injuries. The search continues for alternative methods that complement the arsenal of surgical repair techniques and can provide a rapid and reliable primary repair of transected nerves.

Laser welding

Laser welding is another alternative suggested in several surgical fields to reconnect biological tissues without introducing foreign material into the anastomotic site. Laser welding is suited for applications in which suturing and stapling are particularly difficult, such as in microsurgery or for the treatment of extremely thin tissues (68). The process is based on a photothermal interaction of laser light with the main components of the extracellular matrix of the connective tissues. Heating of the tissue induces formation of new bonds, resulting in fusion of the opposing tissues at hand. This technique has proved to have several advantages compared to conventional suturing methods. The advantages include: reduced operation times, watertight closures, decreased foreign-body reaction with a reduced inflammatory response, faster healing times, and an improved cosmetic appearance (69-70). Laser welding also has the advantage of avoiding traumatising needles used to achieve tissue coaptation. Despite extensive experimental tests on different types of tissues performed during the last few decades, this approach has been clinically employed only in a few surgical applications (71).

Various techniques have been described regarding experimental laser welding of nerve tissue. Circumferential welding consists of creating a stabilising sheath with epineural or local subcutaneous tissue around the united nerve endings to supplement the laser welding (72-73). As an alternative, red blood cells are utilised and placed around the anastomotic site and coagulated with a laser beam (74). Studies have reported favourable use of laser repair, resulting in less scar tissue and reduced constriction at the repair site (75) and, additionally, less time consumption compared to microsuturing (76). However, several authors have reported higher rates of dehiscence after laser repair compared to sutures (73, 77). Furthermore, laser repair requires precise control of the thermal effects to allow welding of the epineurium without damage to the underlying axons.

Due to the problems of decreased tensile strength and an increased dehiscence rate, welding has not been accepted as a superior method compared to microsuturing, although the use of techniques that create a circumferential sheath around the repair site could have certain advantages by preventing fibroblasts from invading the repair site or axon sprouts from escaping.

Fibrin adhesives

As early as the 1940s experimental nerve repairs were performed in which coagulated blood plasma fortified with fibrinogen from cockerels was used to join cut endings of sciatic nerve in rabbits (78). The results were interpreted to yield superior fibre alignment and faster growth across the repair site compared to sutures. However, when

the results were examined, it became clear that various fibrin glues are associated with a range of disadvantages. Some of the drawbacks demonstrated in previous studies include: deterioration in conduction velocity and impairments of the morphometric parameters if regeneration had been completed, as well as a higher cellular response rate compared to sutures (79-80). However, a major advantage has been the considerable gain in operating time compared with conventional microsuturing (80). By now, in clinical practice, fibrin adhesive has become a frequently used tool in the treatment of peripheral nerve injuries in spite of the fact that its cohesive strength has been debated by many authors, and it seems to be insecure during the mechanical healing phase (79, 81). Cruz et al. showed that the addition of fibrin glue to sutures induced greater scar formation with a more pronounced distal axonal disorganisation. In addition, he found 80% disruption when fibrin glue was used alone in repairing a peripheral nerve injury (82). According to Wieken et al. (83), a tension-free repair is necessary with the use of fibrin adhesive, or gaps may appear.

To avoid gaps in the repair site, the nerve anastomosis made with standard fibrin adhesive is often completed in practice with microsutures. However, there are also studies that have demonstrated conflicting results. A modified fibrin adhesive (Tisseel® Duo) has been shown to be comparable to microsurgical anastomosis in tensile strength and morphology (84). Although fibrin glue has been reported, both experimentally and clinically, to decrease operating time, little conclusive evidence has been given of its benefits compared to conventional microsuture repair. Fibrin adhesive is manufactured from blood and is therefore associated with risks of transmission of infective blood-borne diseases. The risk of viral transmission from fibrinogen and thrombin recently halted further developmental work on fibrin sealants in the United States (85). Commercially available fibrin glue (Tissucol® or Tisseel®) is made up of a two-component system. Mixing of the two components takes place immediately before use. Shortcomings which prevent the acceptance of fibrin glue as a full-fledged surgical tissue adhesive include: short-term persistence (< two weeks) *in vivo* and low viscosity prior to polymerisation with thrombin, which make the adhesive difficult to apply (83, 85-86).

Cyanoacrylates

Cyanoacrylates (CAs) were first synthesised by Ardis in 1949 (87). A decade later, Coover realised the potential benefits in the medical field and suggested their possible use as a surgical adhesive in 1959 (88). CAs are synthetic esters of cyanoacrylic acid that rapidly harden by polymerisation on contact with weak basic substances such as air, water, or blood, which makes the compound extremely adhesive to biological tissue (89). The polymerisation of CA is exothermic while forming a thin solid seam between the tissues, and it continues to retain its adhesive qualities even in the presence of moisture. Furthermore, in biological applications, these adhesives have shown a distinguishing quality of being bacteriostatic and haemostatic (90-91). In wound closure they have incurred an additional benefit of being much faster than traditional suturing (92). CA can be easily applied as a single-component glue system conveniently stored in a tube (93). The adhesives are inexpensive, do not carry any risk

of viral transmission, and have sufficient strength to maintain, for instance, a nerve anastomosis, even under tension (94).

Hydrolytic degradation of CA polymers in a physiological milieu yields, however, toxic by-products such as alkylcyanoacetate and formaldehyde, which are further metabolised and excreted in urine and faeces (93). The degree of local toxicity is influenced by the rate of elimination, which depends on the vascularity of the surrounding tissues. When the alkyl chains of the molecular structure increase the amount of carbon, the compound degrades more slowly, resulting in less toxic levels of the by-products compared with derivatives of cyanoacrylate with short alkyl chains (95), *e.g.* methyl 2-cyanoacrylate which was the first CA to be used as an alternative to sutures for the closure of surgical wounds (96). The tensile strength is, however, generally less with longer chain derivatives (97).

The cytotoxicity of CA adhesives in surgical applications has been debated by several authors, however. When used as a tissue adhesive, CA has been shown to induce a stronger tissue reaction than non-resorbable sutures, resulting in a more pronounced foreign-body inflammatory reaction, and it has been shown by others to cause tissue necrosis *in vivo* (98-99). In some reports the inflammatory reaction around the repair site has been shown to be harmful (83) and to cause a focal hindrance to the recreation of tissue (65). However, in other studies, the inflammatory reaction has been shown to require active communication between the injured tissue and the recruited macrophages required for degradation and tissue regrowth and, consequently, to be an integral part of the healing process (100-101).

Efforts have been made to decrease the toxicity by adjusting the rates of viscosity, polymerisation, and degradation and to improve the spreading characteristics, by modification of the chemical structure to make it suitable for different clinical demands. These modifications have resulted in CAs with different bonding properties and CA adhesives have now been used in several different medical fields (102). In its most common role, CA has primarily been used as a tissue adhesive for surgical and traumatic wound repair. As such, it has often been very successful. There are several examples of other surgical and medical applications: in general surgery for haemorrhage control and embolotherapy of various vascular malformations (103-104); in ophthalmology for retinal or corneal repair (97); in thoracic surgery for closure of pulmonary leaks (105); in otological surgery for ossicular chain (*i.e.* the small bones of the middle ear) reconstruction (106), etc. In addition, the *in vivo* degradation potential of CA and of its good acceptance by living tissues has made the adhesive suitable as a drug carrier in the field of pharmacotherapeutics.

A commercially available synthetic adhesive for primary nerve repair does not yet exist, exist. Knowledge concerning nerve regeneration after nerve repair with cyanoacrylate is still largely non-existent, however. In an effort to get maximum tensile strength with optimum viscosity, ethyl-cyanoacrylate (Evobond®), a commercially available ECA with a minimum of additives, was chosen for the studies included in this thesis. ECA has the chemical formula $C_6H_7NO_2$.

AIMS OF THE STUDIES

The present experimental studies were undertaken in an attempt to evaluate cyanoacrylate adhesive as an alternative to sutures in the repair of a transected peripheral nerve.

The specific aims were:

Study 1

to find out if repair of a transected peripheral sensory-motor nerve with cyanoacrylate adhesive was equivalent to conventional microsurgical suturing by measuring physiological and morphological variables after reinnervation is completed.

Study 2

to evaluate the occurrence of macrophages and the distribution of injured axons adjacent to the repaired site after nerve repair with ethyl-cyanoacrylate, and to compare the results with those of conventional microsuturing after a short postoperative period as well as at the time when reinnervation is completed.

Study 3

to compare the possible cytotoxic effect of ethyl-cyanoacrylate on the human neuroblastoma cell line SH-SY5Y with the effects of butyl-cyanoacrylate (Histoacryl®), an adhesive approved for skin closure.

Study 4

to compare two different repair techniques, microsuturing vs. coaptation with ethylcyanoacrylate (ECA), in the process of reinnervating the lateral gastrocnemius muscle (LGC) following sciatic nerve transection and subsequent repair.

MATERIALS AND METHODS

This section gives a brief account of the methods used. For further details, the reader is referred to the individual papers.

Animals

For experiments in *papers I, II, and IV*, female Sprague-Dawley rats weighing 180–200 g were used. The rats were anaesthetised with an intraperitoneal injection of Hypnorm vet.® (fentanyl 0.05 mg/ml and fluanisone 2.50 mg/ml, Janssen Animal Health Ltd) 0.4 ml/kg intraperitoneally and midazolam 2 mg/kg intraperitoneally. To compensate for loss of fluid during anaesthesia, the rats were given a subcutaneous injection of saline (2 ml; 0.9 mg/ml NaCl) every 30 min. During surgery, the rats were kept on a heating pad. All animals in the experiments were used according to a protocol approved by the Ethics Committee for Animal Research in southern Stockholm.

Synthetic adhesive

The ethyl-cyanoacrylate adhesive (ECA) used in this study is commercially available (Evobond®, Tong Shen Enterprise Co., Ltd., Taiwan) and supplied in a 2-ml plastic ampoule with a stiff-tip applicator at one end. In this configuration, it was impossible to apply the adhesive during the microsurgical repair procedures (*papers I, II, and IV*). For this reason, a couple of drops (0.2 ml) of the adhesive were transferred into a 1-ml syringe with a needle. A minimal amount of the adhesive was applied gently to the proximal nerve ending after transection. ECA has the chemical formula $C_6H_7NO_2$.

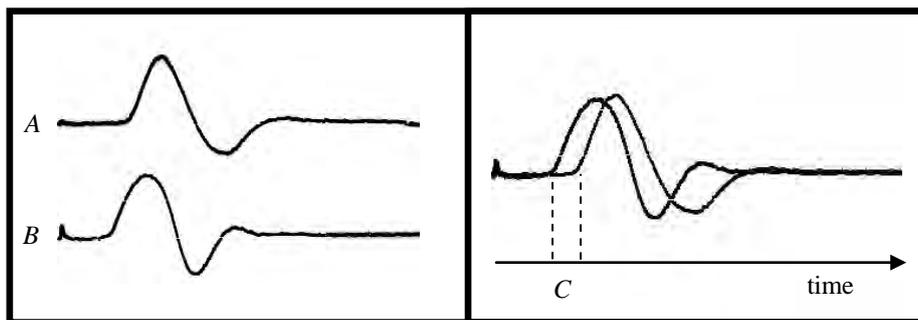
Sciatic nerve injury and repair

In *papers I, II, and IV* experiments were performed using the rat sciatic nerve, either unilaterally or bilaterally. When nerve transection and repair were performed, the left sciatic nerve was used exclusively. If experiments were performed bilaterally, the right sciatic nerve was used as a control. The nerve was transected at mid-thigh and readapted microsurgically with ECA or epineural sutures, with an equal number of animals divided into two groups in each study. In the adhesive group the nerve injury was repaired with a minimal amount of the adhesive applied gently to the proximal nerve ending. The two nerve endings were then carefully brought together microsurgically with two pairs of microforceps. On application the adhesive polymerises instantly to form a thin polymer film that adheres to the opposite nerve endings. Other rats had their transected nerve repaired using three 9/0 monofilament interrupted nylon sutures (Ethicon, J & J, Somerville, NJ, USA) evenly spaced in the epineurium.

Electrophysiology (*Paper I*)

Six months postoperatively the tibial branch to the lateral gastrocnemius muscle and the caudal sural cutaneous nerve were examined bilaterally with electrophysiological measurements. The motor conduction velocity (MCV) across the repair site was assessed by stimulating the sciatic nerve proximally and distally to the repair, with recording and reference needle electrodes placed in the muscle. The latency of the onset and the maximal baseline to peak amplitude of the evoked compound motor action potential (CMAP) were recorded and displayed in an oscilloscope (see Figure 1). MCV was calculated by dividing the distance between the proximal and distal sites of stimulation by the difference in conduction time. The sensory conduction velocity (SCV) was calculated by stimulation to the sensory nerve distally and recording the sensory nerve action potential (SNAP) proximally. The conduction time was measured and SCV was calculated by dividing the distance by the conduction time. Motor nerve action potentials were examined by measuring the maximum baseline to peak amplitude of the CMAP.

Figure 1



MCV across the repair site were assessed by stimulating the nerve proximally (A) and distally (B) to the repair, with recording electrodes placed in the muscle. MCV was calculated by dividing the distance between the proximal and distal sites of stimulation by the difference in conduction time (C).

Histology (*Paper I*)

After the electrophysiological analyses, the tibial branch of the lateral gastrocnemius muscle and the caudal sural cutaneous nerve were carefully dissected and transected bilaterally, each specimen being roughly 6 mm long. The animals were killed with an intraperitoneal overdose of pentobarbitone sodium and ethanol (Apoteksbolaget). The specimens were fixed by immersion in 2% glutaraldehyde in PBS, postfixed in 2% osmium tetroxide, rinsed and dehydrated in graded series of acetone and ethanol, and embedded in epoxy resin (Durcupan ACM (Fluka®)). Cross-sections of the regenerated structures were cut with an ultratome, stained in a 1% solution of toluidine blue and examined by light microscopy.

Morphometry (*Paper I*)

Sections without artefacts were chosen after examination under a light microscope. Microscopic images were captured with a digital camera, edited in Adobe® Photoshop® CS software (version 7.0; Adobe Systems Inc., San Jose, CA, USA) and analysed using morphometric software (ImageJ 1.33u, National Institutes of Health, USA). The following morphometric indices were calculated for each nerve: mean cross-sectional diameter of the myelinated axons, fibre density (number of myelinated axons/mm²), and total number of myelinated axons.

Immunohistochemistry (*Papers II, IV*)

Immunohistochemistry was performed to detect neurofilaments, macrophages, neuronal growth cones (*paper II*), α -motoneurons and presynaptic structures (*paper IV*). In *paper II*, at seven days after reparation and after six months, when reinnervation was completed, segments of the sciatic nerves with the repair site centred, as well as opposite sciatic nerves at corresponding sites, were excised. The specimens were freeze-sectioned longitudinally and mounted on slides. Sections were then air-dried and soaked in PBS. Incubations were performed in a humid chamber with either of one of the following primary antibodies: a mouse monoclonal antibody against rat macrophage surface protein (ED-1, Serotec Antibodies, Sweden, dilution 1:500), a mouse monoclonal antibody against neurofilament (Pan/NF, Zymed Laboratories, Inc., CA, USA, dilution 1:200), a goat polyclonal antibody against Growth Associated Protein 43 (GAP-43, Santa Cruz-7457, SDS, Sweden, dilution 1:100) and, finally, a goat polyclonal antibody against the glucose transporter Glut-1 (Glut-1, Santa Cruz-1605, SDS, Sweden, dilution 1:100). Sections were also double-labelled with different sets of compatible combinations of the above-mentioned antibodies. In *paper IV*, after the specimens were frozen-sectioned transversally, incubations were done similarly, but with the primary antibody, goat anti-CTB (List Biological Laboratories, Campbell, CA, USA, dilution 1:1000). In the cases of double labelling, sections were labelled using two different fluorophores and processed for CTB immunohistochemistry as described above, combined with immunoprocessing with one of the two following cellular markers: rabbit anti-CGRP (Bachem, Bubendorf, Switzerland, dilution 1:400), mouse anti-Synaptophysin (Sigma, Saint Louis, Missouri, USA, dilution 1:400). In *paper II* incubation with secondary antibodies was carried out, using either Cy3-donkey anti-mouse conjugated IgG (Jackson ImmunoResearch, Inc. PA, USA, dilution 1:400), Cy2-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, Inc. PA, USA, dilution 1:200), or mixtures of them depending on the primary antibodies in the double-labelled sections. In *paper IV* incubation with secondary antibody was carried out, using Cy3-conjugated donkey anti-goat IgG (Jackson Immuno Research, PA, USA, dilution 1:1000) and in the cases of double labelling, sections were incubated with Cy2-conjugated donkey anti-rabbit IgG (Jackson Immuno Research, PA, USA, dilution 1:200) or donkey anti-mouse IgG (Jackson Immuno Research, PA, USA, dilution 1:200). Subsequently, the sections were rinsed and mounted in a 1:3 solution of glycerol and PBS.

Retrograde tracing (*Paper IV*)

Six months postoperatively the tibial branch of the lateral gastrocnemius muscle (LGC) of all experimental animals was cut at a location just before it entered the muscle. The proximal nerve ending was inserted into a polyethylene capsule containing a solution of 1% cholera toxin subunit B low salt (CTB; List Biological Laboratories, Campbell, CA, USA) for 30 minutes. The opposite side was processed by the same method and used as a control. Three days later the animals were perfused and the L5 spinal cord segment was removed, post-fixed and cryoprotected in sucrose. Frozen cross-sections were cut at 14 μm and mounted sections were processed for single and double labelling.

Image processing and analyses (*Paper II, IV*)

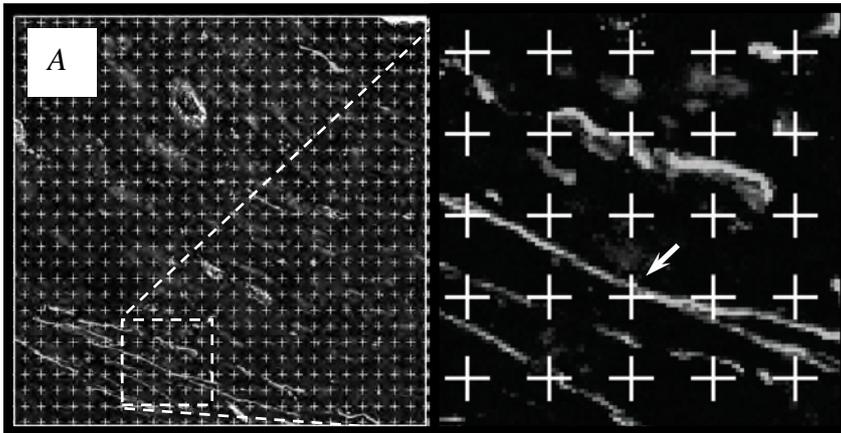
In *paper II* the specimens were examined under a light microscope equipped with epifluorescence and appropriate filter combinations for the fluorophores used. Fluorescence- microscopic as well as conventional bright field images, with identical visual fields and magnification, were digitally captured. Micrographs were imported into and edited in Adobe Photoshop® and analysed using morphometric software (ImageJ/NIH). To analyse the relations between the remaining adhesive or sutures and the labelled tissue structures, bright field images that visualised only the adhesive, elucidated with a phase contrast filter, were superimposed on the fluorescence microscopic images. Representative fields were then chosen. In *paper IV* the specimens were examined using a fluorescence microscope and images were digitally captured and edited in Adobe Photoshop®. Quantitative (using morphometric software [ImageJ/NIH]) and descriptive analyses of labelled α -MNs were performed bilaterally.

Confocal microscopy (*Paper II, IV*)

In *paper II* neurofilaments and ED-1-positive macrophages, proximal and distal to the repair site, were assessed quantitatively with laser scanning confocal microscopy for each specimen. Corresponding analyses were made on uninjured sciatic nerves from the control sides. In *paper IV* synaptophysin-immunoreactive (ir) profiles in the immediate vicinity of RLMs were assessed quantitatively in the lateral ventral horn. Representative sections were chosen from the middle of the nerve trunk (*paper II*) or from selected sections from the spinal segment L5 (*paper IV*). Standardised square fields were chosen in the fluorescence microscope and the quantitative morphometric examinations were optimised by reproducing each square field in a microscope equipped with a laser scanning confocal system. Fluorescence was through a green or a red filter, respectively. Stacks of 14 optical serial confocal sections taken at 1.05- μm intervals (*paper II*) or stacks of three optical serial confocal sections taken at 0.15- μm intervals (*paper IV*) were reconstructed to yield two-dimensional digitised images. In *paper II* the ratio occupied by the labelled structure of interest was estimated by placing a grid of systematically positioned test points (crosses) randomly on each two-dimensional digitised image using ImageJ/NIH. The number of grid points that fell into

the labelled structures divided by the total number of grid points formed the area ratio estimated (see figure 2). The ratio of labelling occupied by the structure of interest was expressed as a percentage. In *paper IV* the two-dimensional digitised images were captured as TIFF files and imported into ImageJ/NIH. For each RLM (repair and control side), the total perimeter of the cell body outline was estimated and the total extension of the sections of the outlined circumference occupied by synaptophysin-immunoreactive profiles was measured. The ratio occupied by the labelled structure of interest was then calculated, the mean value for each specimen was also calculated, and the percentage of repaired versus control side was generated, followed by quantitative comparisons (%) of the two repair methods.

Figure 2



The ratio occupied by the labelled structure of interest was estimated by placing a grid of systematically positioned test points (crosses) randomly on the two-dimensional digitised image (A). The number of grid points that fell into the labelled structures (*arrow*) divided by the total number of grid points formed the area ratio estimated.

Muscle weight (*Paper IV*)

The wet weight of the LGC was determined using an electronic balance and expressed as a percentage of the contralateral unaffected side to correct for individual differences.

Cell culture experiments (*Paper III*)

Exposure to adhesives

SH-SY5Y cells, a human neuroblastoma cell line, obtained from ATCC (American Type

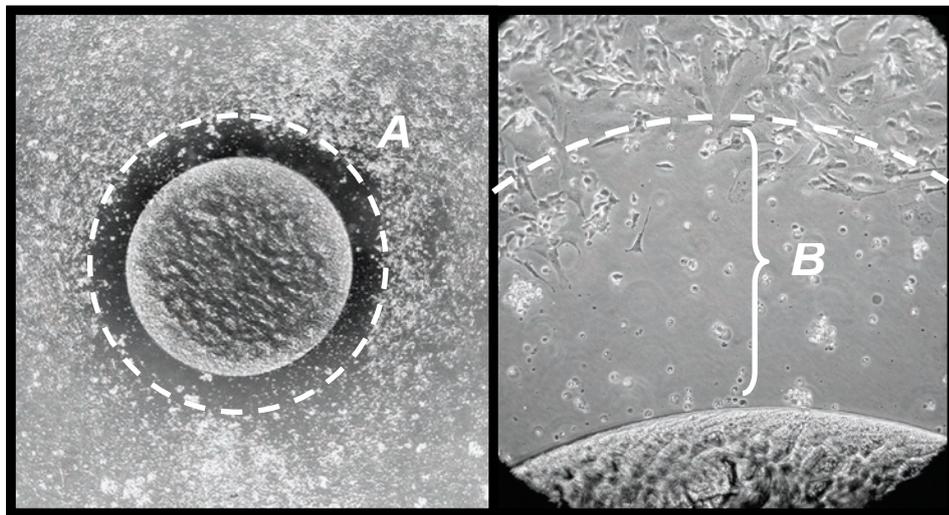
Culture Collection, Manassas, VA, USA) were used in this study. For further details regarding preparation and analyses, see *paper III*. Briefly, SH-SY5Y cells were cultured on the bottom of 24-well polystyrene culture dishes. At confluence, monolayers of cells were exposed to the adhesive by adding 0.1 μl of ethyl- or butyl-

cyanoacrylate to the centre of each well. Cell cultures were followed up for up to 28 days after exposure and at predetermined time points the cell viability was examined using the following methods:

Evaluation of the halo devoid of cells

At the predetermined time points, microscopic images were captured digitally from the cultures by phase contrast microscopy (Nikon Diaphot 300, Nikon Corp., Tokyo, Japan) with the polymerised adhesive droplet centred. All micrographs were displayed on a computer monitor and analysed using morphometric software (ImageJ/NIH). The outer edge of the cell-free halo around the adhesive was approximated to a circle and the distance from the adhesive border to the cell layer margin was measured (see figure 3).

Figure 3



The outer edge of the cell-free halo (A) around the adhesive was approximated to a circle and the distance (B) from the adhesive border to the cell layer margin was measured.

Detection of cell death

Cell death in this study was assessed using a ^{51}Cr cytotoxicity assay, which is an established method for quantification of cell toxicity (107-108). As described in *paper III*, cells (in exposed cultures as well as controls) must be labelled with ^{51}Cr (Perkin Elmer, Boston, MA, USA) before each predetermined time for detection. Immediately after exposure to the adhesives and at predetermined time points, detached cells were separated from the supernatant by centrifugation. The remaining SH-SY5Y cells in each well were lysed with NH_4OH . The radioactivity of the supernatants and the remaining SH-SY5Y cells was measured in a gamma counter. Cell death, expressed as a percentage of ^{51}Cr release, was calculated as follows:

$$^{51}\text{Cr release} = A/(A + B) \times 100$$

A stands for mean counts per minute in the supernatant and B, mean counts per minute from the remaining SH-SY5Ys. Finally, the specific ^{51}Cr release, *i.e.* ^{51}Cr release due to the adhesive, was obtained by subtracting the mean ^{51}Cr release in controls from the mean ^{51}Cr release in any exposed well.

Analysis of cell morphology

A cytoskeletal assessment was made 24 h and three and seven days after exposure. The staining method for tubulin has been described in detail elsewhere (109). The specimens were examined by fluorescence microscopy (Nikon Eclipse E600, Nikon, Tokyo, Japan) and photographed with a digital camera (Nikon Digital Sight DS-U1). Images were captured from each culture in four different quadrants perpendicular to each other (total of four images per cell culture) from the area immediately outside the cell-free halo (see above). Five separately located cells (displayed on a computer monitor) were selected randomly from each micrograph and cell sizes were analysed using morphometric software (ImageJ/NIH). Furthermore, cell bodies and neurites were analysed descriptively with respect to shape and texture at all time points.

Statistical analyses

Results are presented as the mean \pm standard deviation (SD). Probabilities of less than 0.05 were accepted as significant. Comparison by analyses of variance (ANOVA) were made to find out if there were any differences between mean values in the groups (*papers I, II, IV*). The normality distribution of each variable was calculated using the Kolmogorov-Smirnov test before comparisons of morphometric data among the different repair techniques were made using Student's unpaired t test. In *paper III* differences within and between groups were analysed using a Mann-Whitney test, one-way ANOVA, or repeated measures ANOVA when appropriate. All statistical tests were performed using the software GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). The statistical analyses of the electrophysiological data in *paper II* were confirmed using the Statistical Package for the Social Sciences, version 12.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

The following is a summary of the results and some comments. For in-depth details, please refer to each individual paper.

Paper I

Peripheral nerve repair with ethylcyanoacrylate supported functional and morphological recovery

Our objective here in this first study was to test the hypothesis that a synthetic ethylcyanoacrylate adhesive, when used by itself, can measure up to the standards of conventional microsurgical suturing. The focus was concentrated on the recovery after repair of a transected peripheral nerve.

Six months after a unilateral lesion of the sciatic nerve and subsequent repair, the tibial branch to the lateral gastrocnemius muscle and the caudal sural cutaneous nerve were examined with electrophysiological measurements of motor and sensory conduction velocity, motor nerve action potentials, and quantitative histological examinations. The regenerated anastomoses were observed and showed similar results in all animals within both groups. The repair sites after repair with ECA were, however, less hypertrophic than those repaired with microsutures. Remnants of suture material were found at each repair site within its group, whereas no residue of ECA could be found within the other group.

Electrophysiological findings

Both groups were compared with the control side. Reduced values of MCV as well as SCV were noted after repair regardless of which method had been used. The mean MCV was significantly higher in the group repaired with ECA than in the sutured group. The mean values for SCV were slightly higher in the group repaired with ECA. This difference was not significant, however. In all rats, stimulation of the repaired sciatic nerve evoked CMAPs with amplitudes that were consistently lower than in the intact opposite nerve. No differences were found after comparing the mean values of CMAP amplitudes and the mean ratio between the two groups.

MCV and SCV, which are dependent on the diameters of axons, myelination, and internodal distance (110), are commonly used physiological indicators that correlate with functional recovery of regenerated nerves. The amplitude of the CMAP is a reliable indicator of the number of muscle fibres innervated by regenerated axons. Mean nerve conduction velocities for both methods were 70% - 80% of control values six months after injury, which according to the sutured group is well in accordance with recent reports using a similar experimental design (111), but with a significant increase in MCV recovery but no significant difference in SCV after ECA sealing. No significant differences between the two methods were found when mean values of CMAP amplitudes were compared. The dispersion in CMAP amplitudes was large for both groups, which is probably at least partly the result of difficulties in obtaining

exactly the same position of the recording needle between the different animals. This might obscure small differences in mean amplitudes between the two methods.

Histological findings

Light microscopic examination of histological sections distal to the repair site showed regenerated myelinated axons in both groups. A large number of myelinated axons were observed in all nerves, in both the tibial branch to the lateral gastrocnemius muscle and the caudal sural cutaneous nerve. Both repaired groups had smaller axonal diameters, increased fibre density, and an increase in total number of myelinated axons compared with control nerves. None of the morphometric variables differed significantly between the two groups.

The total number of myelinated axons, cross sectional diameter of myelinated axons, and nerve fibre density (reflecting reinnervation and nerve maturity (110)) showed no significant difference according to the quantity and quality of regenerating axons for the two methods. For all repaired nerves the calculated changes in the morphometric variable decreased, except for the number of myelinated axons, which increased. Excessive numbers of myelinated axons distal to the site of a nerve injury after repair has been well described by, for example, Mackinnon et al. (112).

Paper II

Ethylcyanoacrylate seems to induce propitious local tissue reactions on nerve regeneration

In this, the second study, we examined the reactions of the local tissue after transection and repair of the sciatic nerve in a rat. A comparison was made between suture repairs and those made with synthetic ethylcyanoacrylate adhesive. The occurrence of macrophages and the distribution of injured axons adjacent to the repaired site were evaluated after a short postoperative period and, again, at the time of completed reinnervation.

Gross examination

Seven days after repair: Adhesions to the surrounding muscles were more pronounced in the group of adhesive-treated rats than in the group treated with sutures.

Six months after repair: The sites of injury after repair with ECA were less hypertrophic than those that had been repaired with microsutures. Some suture material was seen at the repair sites of the group repaired with microsutures. No adhesive residue could be seen in the other group.

Immunohistochemistry

ED-1 (Macrophages)

Seven days after repair: Quantitative data showed a significant increase in ED-1-ir profiles proximally and distally to the repair site with ECA compared with epineural sutures. There was a strong labelling for ED-1 at the adhesive interface all along the proximal cross-line, although this was somewhat less pronounced along the distal

cross-line. In the sutured cases there was a slight accumulation of ED-1-ir around the remnants of the microsutures.

Six months after repair: The area of ED-1-ir profile distribution was narrower, and ED-1-stained cells were less abundant compared to short survival time. Remnants of ECA within the regenerated nerve were surrounded by thick layers of ED-1-labelled cells. Microsutures, which could be seen in only a few cases, were also surrounded by ED-1-ir cells, but the thickness of the layers was less pronounced in these cases than in those treated with ECA. Quantitative data showed a slight, but significant, increase in ED-1-ir profiles around the repair site six months after repair with ECA compared to epineural sutures.

Several reports have confirmed that recruited macrophages have a role in degeneration of peripheral nerve axons (101) as well as in degrading and removing myelin [26] after a nerve has been injured. Macrophages also have an important role in the regeneration process because of their ability to secrete a vast range of growth-promoting products (101, 113). However, the mechanism of recruiting macrophages in Wallerian degeneration is still not clear, and knowledge about the regulatory mechanisms and signalling cascades that underly the complex molecular regeneration program is still limited (114). The accumulation of the ED-1-stained macrophages was confined to the part of the transected nerve that was devoid of Glut-1-ir perineurium, which suggests that the recruitment of macrophages in Wallerian degeneration needs a breakdown of the blood-brain barrier. Cyanoacrylate causes a more intense foreign-body inflammatory reaction than conventional non-resorbable sutures when it is used in biological tissue (83, 98, 115). Our observation that the number of ED-1-stained cells, which are considered to reflect the number of activated macrophages, were increased around the repair site in the adhesive group, suggesting that the presence of cyanoacrylate stimulates the recruitment of macrophages. Compared with sutures, cyanoacrylate repair increased the distal breakdown of axons (see below), indicating an accelerated Wallerian degeneration as a result of an enlarged population of macrophages. Experimentally-induced inflammation by application of a foreign body that attracts macrophages has been shown by others to stimulate nerve regeneration (116). It has also been shown that it is possible to impair the regenerative ability of axons by preventing the invasion of macrophages (117). An adhesive that stimulates an immediate inflammatory reaction could therefore produce a quicker regenerative response.

Our findings that aggregates of ED-1-positive macrophages were covering most of the solid parts of ECA seven days after repair and likewise, remnants of ECA were embedded by thick layers of ED-1-positive macrophages after six months, indicates that those recruited macrophages have an active role in the elimination of the adhesive presumably by phagocytosis making the adhesive fragmented. The difference in the number of ED-1-positive macrophages between the suture and the adhesive group still remaining at six months is probably the result of a foreign-body inflammatory reaction against remnants of ECA scattered within the repair site. This excess is, most likely, temporary and when the degradation and elimination of ECA is completed the macrophages will probably vanish.

PAN/NF (neurofilaments)

Seven days after repair: In longitudinal sections of the cases treated with adhesive, the axonal alignment of the neurofilaments was well structured. This could be concluded to be due to the fact that they were parallel to the nerve axes, right to the interface of the adhesive.

Within the sutured nerves, the neurofilaments were found to be disorganised in proximity to the repair site; most of the axons were misdirected and a number of them were found to be located outside the main nerve trunk. The poor orientation in the midst of the repair site with many axons cut transversely were giving the impression that nerve endings had been forced together while repaired. Quantitative data showed a significant decrease in neurofilament-stained profiles distal to the repair site in the adhesion cases compared to the sutured ones, but there was no difference proximally.

Six months after repair: Staining for neurofilaments was less apparent in all regenerated nerves than in the controls. This held true regardless of which method of repair had been used.

GAP-43 (path finding growth cones)

There were numerous thin and branching structures proximally to the repair site, clearly expressing GAP-43-ir, with no obvious difference between the two repair methods.

All of the samples in the ECA cases had a thin stripe of cyanoacrylate, occasionally fragmented, between the anastomotic ends with the alignment of the neurofilaments well structured right into the interface of adhesive. Nerve regeneration was observed starting in the proximal stump, with pathfinding axons beginning to penetrate the ECA barrier, indicated by numerous GAP-43-ir fibres in close contact with the proximal border of the adhesive. These findings in combination with observations of no evident differences between the two repair methods with respect to the number or distribution of neurofilament-positive profiles indicate that no acute axonal degeneration caused by the adhesive has been taken place proximal to the seam. Absence of GAP-43-ir distal to the injury site indicates that the regenerated axons by two weeks after transection not yet have grown across the repair site.

Paper III

The transient nature of the cytotoxic effect of cyanoacrylate in nerve repair:

In this study we used SH-SY5Y, a widely employed neuronal cell line for studying neuronal toxicity and neurocytoprotection (118). For comparison, we studied the cytotoxicity of Histoacryl® (butyl-2-cyanoacrylate), which is considered to be one of the least cytotoxic cyanoacrylate derivatives and, consequently, one of the most frequently used CAs in general surgery.

Adhesive-induced cell death measured with ⁵¹Cr

The first two weeks: The two adhesives induced rapid cell death during the first 24 hours, which decreased dramatically during the first two weeks of exposure.

Weeks 2, 3, and 4: From the second to the fourth week, the amount of cell deaths decreased further, but now at a slower pace.

At 28 days: No significant differences in toxicity were seen between exposed wells and the controls. Also, there was no noticeable difference in cell toxicity between the two adhesives during any time period of this experiment.

Using a ^{51}Cr release assay, we demonstrated a significant, but transient, cytotoxic effect of ethyl-cyanoacrylate as well as butyl-cyanoacrylate immediately after exposure to the nerve cells. This is in conformity with the study by Thumwanit and Kedjarune (119) which investigated the effect of a mixture of ethyl- and methyl-cyanoacrylate on cultured oral fibroblasts and showed a considerable reduction in cytotoxicity during the first 24 hours, followed by a continuous decrease in the inhibitory effect during the following studied two weeks. In our model, maximum cytotoxicity was observed at 24 hours and significant cytotoxicity was seen up to seven days after exposure, whereupon it was almost negligible after a time delay of two weeks from exposure.

Adhesive-induced halo devoid of cells

Twenty-four hours after incubation: At this point, there was evidence of a cytotoxic milieu in conjunction with the adhesive border, which produced a halo without cells. This halo was slightly wider (although not significantly) in the ECA group when initially compared to the BCA group.

The first two weeks: The cell free zones expanded considerably during the next seven days. Within both groups these zones subsided significantly between days 7 and 14.

Weeks 3 and 4: Between 14 and 28 days after incubation, the halos continued to diminish. At 28 days, cells had reached the margin of the adhesive in the ECA group, which at this point made the cell-free zone barely measurable. However, the cell-free zones in the BCA group were noticeably wider than those of the ECA group by the end of the study period.

During the first week after exposure the halo increased, but then gradually diminished during the following three weeks, which, in contrast to the ^{51}Cr release method, indicated maximum cytotoxicity after one week. This time delay for the morphometric assay may be explained by the fact that the ^{51}Cr assay measures cytotoxicity at a specific time point, while the halo is also a result of prior cytotoxicity. At the end of the observation period, cells in the ECA group were found to reach the margin of the adhesive, evidently growing independently of the presence of the adhesive. In the BCA group, a small but visible distance between the adhesive and cells remained throughout the study period, indicating a persistent cytotoxic effect. This could not be confirmed, however, by the ^{51}Cr method. Although the ^{51}Cr release method is known to be highly sensitive (120), it is difficult not to ascribe this phenomenon to a sensitivity problem. Since Thumwanit and Kedjarune (119) did not follow the cell line for more than two weeks, no conclusion about the cytotoxicity beyond this time point can be drawn from their study.

Adhesive-induced changes in cell morphology

Basal cytotoxicity has also been evaluated *in vitro* by studying neurodegenerative properties in SH-SY5Y neuroblastoma cells during exposure to toxic substances (121-122). In our study, a complete degeneration of neuritis, cell shape changes, and texture

degeneration were seen in all cultures 24 hours after exposure, regardless of which adhesive had been used. Moreover, groups of cells formed clusters, which were less evident at farther distances from the adhesive. Cells located in the area close to the cell-free halo exhibited a significantly *smaller quantity* compared to the control wells. Signs of recuperation were seen after three days were cells had regained their shape, with neurite outgrowth being frequently observed. This was seen to be rougher, however, and was of shorter extension than the control wells. At seven days after continuous exposure to the adhesive, a total recovery of the neuroblastoma cells, with morphology equal to that of controls, could be demonstrated.

No differences in cell morphology, descriptive or quantitative, were observed between the two adhesive groups at any point. These findings are in line with the results of the ^{51}Cr and morphometric assays and further support the conclusion that the cytotoxic effect of CA is of a temporary nature (123).

Paper IV

Selectivity of motor reinnervation after nerve repair with ECA (is comparable to that following conventional microsuturing).

In this fourth study our objective was to find out if ECA has a negative impact on the selectivity of motor reinnervation following sciatic nerve transection and subsequent repair. After reinnervation had been completed, six month after repair, retrograde neuronal tracing with single and double-labelling techniques was performed to evaluate quantitatively and descriptively the accuracy of motor axons for regeneration to the original target.

Fluorescence microscopy and morphological examination

In the L5 spinal cord, identified α -MNs were abundantly supplied with dendrites, which were observed with intense immunofluorescence labelling. Nuclei were observed centrally in the cell bodies without any tendency to swelling or a withered appearance. The above-mentioned observations apply to all assessed α -MNs, as well as to the controls, and regardless of which method was used for the repair.

Retrograde tracing of regenerated motoneuron

While studying the quantitative data and comparing to the controls, the anatomical cross-sectional distribution area of RLMs at the spinal L5 level increased slightly more than 100% in both repair groups. Both groups, compared to the control side, also showed an increase in the number of detected RLMs representing the LGC, located in the lateral ventral horn of the L5 spinal cord, whereas the number of RLMs located within the assumed normal anatomical distribution area in the grey matter was slightly more than half of the total number of RLMs distributed on the repair side.

The motoneurons projecting to a specific muscle, scattered throughout a larger volume of the spinal cord grey matter than controls and intermingling within the lumbar ventral horn, suggesting that motoneurons originally belonging to adjacent nuclei aberrantly project to the experimental muscle after regeneration. Valero-Cabr e et

al. showed a spreading of the tibialis anterior nucleus to more ventral regions of the spinal cord grey matter interpreting that motoneurons originally projecting to the lateral gastrocnemius muscles aberrantly reinnervate the tibialis anterior muscle after nerve cut and repair, and vice versa (111). Furthermore, our quantitative results are consistently with Valero-Cabr e et al. finding three motor nuclei from the hind limb significantly enlarged in transversal perimeter after sciatic nerve transection and suture repair. The most marked increase was the anterior-posterior axis of the nucleus of the LGC (111). Our findings with an apparent increase in number, together with a larger myotopic distribution area of retrogradely labeled motoneurons (RLMs), regardless of repair method, can not find consistency in previous reports. However, regenerating axons have been demonstrated to form more than one branch (124) and as a consequence *polyinnervation* i.e. the attachment of more than one motoneuron to the same endplate (125), *hyperinnervation* i.e. the projection of more motoneurons into the same muscle after reinnervation than before lesion (126) and *axonal trunk bifurcation*, i.e. simultaneous axonal branches to more than one muscle by long collateral branches of the same motoneuron (127), contributory factors that greatly might explain the increase in number as well as the enlarged and disorganised myotopic distribution of RLMs when reinnervation had well been completed.

The reduced number of labeled neurons, within the assumed normal myotopic distribution area, is thus an indication of surviving cells reinnervating the motor targets and a reduction after repair may be attributed to axonal misdirection to non-labeled targets rather than to neuronal death. In any case, with a more sparse α -MN population in the assumed normal myotopic distribution area connected to the original peripheral target, follows a presumed decrease in appropriate supraspinal and propriospinal input resulting in reduced functionality in the muscle.

The mean cross-sectional area of RLMs in the lateral ventral horn was similar for the two repair methods. Both, however, had increased slightly compared to the controls, although not significantly so. In most controls, the RLMs appeared to merge so as to form one common column that occupied the described dorsomedial position in the lateral ventral horn of the grey matter. In repaired specimens, regardless of the repair method, the RLMs were scattered irregularly in all directions within the ventral horn, and the appearance of a column, consisting of labelled cells, sharply marked off from its surroundings, could not be observed.

Moreover, an increase in perikaryal size on the repaired side is consistent with previous studies, reviewed by Lieberman (31). However, there is a wide variation in previous reports from other authors on the effects of axotomy on perikaryal size. Some authors have reported that cell dimensions decrease in response to injury (128). Motoneurons whose axons had been prevented from muscle reinnervation were also described to show some atrophy but the majority of reports dealing with this issue have indicated that there is either a transient swelling or no change in the size of motoneuronal perikarya when injured neurons are permitted to regenerate. Swett et al. showed a considerable increase in perikaryal diameters within three month of injury, with perikaryal swelling occurred rapidly after injury and then gradually subsided to sizes closely approaching normal when regeneration well had been completed (129).

With negligible exceptions, the boundaries of cell bodies and proximal dendrites of RLMs were found to be covered by synaptophysin-immunoreactive profiles. These profiles coated the major portion of the circumference, although with an irregular degree of fluorescence intensity and a rough appearance along the outline.

These findings revealed no difference between the two repair methods. And no noticeable difference was revealed either when compared to the controls.

Confocal microscopy for synaptophysin-immunoreactive profiles

Confocal microscopy further confirmed the apposition of synaptophysin-immunoreactive profiles on RLMs, both on their cell bodies and on proximal dendrites. In contrast to fluorescence microscopy, confocal images provided a precise adjustment of the optical slicing of the tissue (approx. 1 μm of thickness) and thereby facilitated quantitative comparisons of synaptophysin expression. No matter which repair method was used, a quantitative estimation indicated some reduction after injury relative to the controls.

Synaptophysin, used in our study, is a presynaptic membrane protein of neurotransmitter-containing synaptic vesicles, and is expressed ubiquitously throughout all synapses of the CNS and PNS (130). Synaptophysin immunostaining is thus a good marker for presynaptic terminals which has been used widely to estimate the increase or decrease of synaptic numbers on identified motoneurons (131). In both our experimental repair groups we found a similar synaptic coverage of labeled motoneurons, however a significant decrease compared to control sides by approximately one fifth. These findings suggests that the existing reinnervated α -MNs in the LGC nucleus, six month after transection and repair of the sciatic nerve, are subjected to an additive reduction (compare to control side) in synaptic inputs which could result in a further reduction in muscle functionality.

Lateral gastrocnemius muscle weight

Based upon visual examination, the lateral gastrocnemius muscles on the operated sides demonstrated noticeable atrophy compared to the contralateral normal limbs. This was further demonstrated by reduced muscle weight on the experimental side, although no statistical difference could be detected between the two repair methods.

From a clinical perspective, complementary functional tests (i.e. walking pattern, foot withdrawal reflex or toespreading reflex tests) could be desirable to increase our understanding regarding the fate of motoneurons after surgical nerve repair with a synthetic adhesive. However, Beer et al. demonstrated muscle weight as a more detailed method to evaluate functional recovery than toespreading reflex testing and thereby justify its use as a functional parameter of motor regeneration (132). In our present study, an obvious reduction in weight of the lateral gastrocnemius muscle (LGC) compared to controls without any difference between the two repair methods was observed six month after repair.

Despite the benefit of emerging discrete differences in regeneration by weighing muscles, measuring the muscle weight as a functional test has some notable limitations and uncertainties. One disadvantageous limitation is that by measuring the muscle

weight alone, only the weight but not the strength of the muscle is measured. Yet, it is discussed controversially whether indirect tetanic tension and muscle weight do correlate. Brunetti et al (133) found no significant correlation between the two parameters, explained by the fact that the muscle atrophy begins quite early and is still not compensated at the end of that study.

On the other hand, Hie et al (134) found a high correlation between the muscle weight and maximum twitch tension in nondenervated, normal, nontrained rats. Yet, the possibility to measure the weight of LGC separately provides a more detailed insight into the motor regeneration than the functional tests; toe-spreading reflex and thereby justifies the muscle weight as a functional parameter in motor regeneration

GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Regardless of whether a motor, a sensory, or a mixed nerve has been injured, a short interval between lesion and repair is desirable. The many reasons for this desired interval have been explained in experimental, as well as clinical, studies.

Neurotrophic factors

Generally speaking, it is an accepted understanding that neurons are dependent on neurotrophic support in sustaining maintenance and survival during their adult life, as well as the regeneration process after injury. In an uninjured neuron, the trophic factors are produced by the target organ and delivered to the neuron by retrograde axonal transport (135). A chain reaction, known as the axon reaction or chromatolysis, will be initiated in the neuronal soma after a peripheral nerve lesion. The disruption in the supply of retrogradely transported neurotrophic factors leads to neuronal cell death (more severe and rapid in sensory neurons than motoneurons (37)) and a lack of regeneration (136). However, this process can be reversed if the axons reinnervate their target organs and regain neurotrophic support (137-138). It has been proposed that early surgical reconnection of the transected nerve endings could mimic an axonometric injury (the continuity of the nerve is intact, but the axons are damaged and need to regrow), i.e. a crush injury, which is associated with less cell death. Primary nerve repair could therefore provide neuroprotection, and early surgical management would thereby counteract the neuronal death (139).

Shortly after injury, attempts at regeneration begin (140) with a cascade of events involving cell signalling molecules and neurotrophic factors (141). When the axon contacts are interrupted the Schwann cells downregulate their normal proteins and convert to a phenotype (142) that upregulates expression of several neurotrophic factors and other compounds leading to Schwann cell differentiation and proliferation in anticipation of the arrival of a regenerating sprout (143-145). A noticeable increase of the nerve growth factor (NGF), produced in the distal segment by the Schwann cells, has been shown to reach its peak within 24 hrs and is maintained at 10- to 15-fold elevated levels for a minimum of two weeks after axotomy (144). Four days after axotomy, the brain-derived neurotrophic factor (BDNF), known to promote the survival and outgrowth of sensory neurons and motoneurons (146), increases to several times the normal level and reaches its maximum 4 weeks later (145). It is apparent that peripheral nerve regeneration is dependent on the secretion of multiple neurotrophic factors, such as BDNF, NGF, and upregulation of appropriate receptors, which behave in manners complementary to each other in a well-designed and timely sequence (147). The area around the lesion and the distal stump have been likened with a “neurotrophic factory”. Furthermore, a prolonged axotomy has been shown to result in limited regeneration due to atrophy of Schwann cells and, consequently, a reduced expression of neurotrophic factors (148).

The effect of axonal damage is further propagated in a retrograde direction from the periphery towards the nerve cell body. This cell body will then start producing stimuli which, in turn, trigger a reaction in the neurons, which promotes the healing process. Axotomised neurons change from a transmission mode to a growth mode and express growth-associated proteins, such as GAP-43, and a number of new neuropeptides, all of which have the potential to support axonal regeneration (32). In axotomised neurons, there is a coordinated upregulation of several genes involved in the regeneration process. These alterations, which help the neuron to change from transmitting mode to growth mode, start as early as 12 hours after injury (149). The upregulation after adult peripheral nerve injury may hereby both promote survival of the injured neurons and contribute to alterations in the cytoskeleton associated with axonal growth (150). In the previously described study, immediate epineurial nerve repair was compared to a repair which was delayed by one week. The results showed a reduction of sensory neuronal loss from 12% to 5% two weeks after axotomy, which suggests a neuroprotective effect of the repair, the strength of which relates to how early surgery is performed (139). Ma et al. compared the effects on neuron survival 16 weeks after primary repair performed 2 days after transection of the C7 spinal nerve. The results obtained after three different types of nerve reconstruction were compared with those in control animals, in which cases mere axotomy was accomplished. Regardless of which repair method was used, primary repair reduced the loss of sensory neurons by 50%, and the motoneuron loss was reduced by 70% (137).

With the realization that a strictly regulated timetable (144-145) regarding how different levels of neurotrophic factors and receptor proteins cooperate both sequentially and concertedly after injury in order to enhance the support which is essential for the regeneration of injured peripheral nerves (143), it is crucial that nerve repair is not delayed.

Neuroma and scar formation

After a peripheral nerve lesion, a gap between the two stumps is immediately established. This is caused by the elastic retraction of the nerve endings. If a nerve repair can be performed within days of injury, it will eliminate a great deal of the problems associated with this gap and the excessive tension of the nerve. The critical factor is the length of the gap between the proximal and distal stumps. If the proximal stump is obstructed from re-entering the distal stump, i.e. when a repair has failed to occur, Schwann cells, fibroblasts, and blood will soon fill the gap, resulting in increased scar formation around numerous regenerating axons. Axons that cannot reach the distal stump are wasted; they may wander into adjacent tissue or become encased in the scar, which invariably forms within the gap between the stumps, leading to neuroma formation. Without treatment this may lead to hyperaesthesia, chronic pain and, possibly, loss of function. If secondary or delayed end-to-end neurorrhaphy is considered, the operation requires resection of the terminal bulbs (neuroma formations) from proximal and distal endings until a recognisable pattern of healthy fascicles is reached.

An attempt to draw the two stumps together might be technically feasible, but certainly not without injurious tension. Therefore, if a tension-free secondary repair cannot be achieved, grafting should be performed. However, even when a graft is used a considerable portion of the regenerating axons is lost across each suture line (37).

Hence, immediate action in order to prevent harmful neuroma and scar formations while the gap can be made up and a tension-free neurorrhaphy can be performed is of great importance.

Retraction of nerve endings

If the nerve is not repaired acutely, there is a considerable risk that the elastic recoil, combined with increased fixation of the nerve endings by fibrosis, will result in an inflexible gap, preventing re-approximation of the two endings. A factor that needs to be considered when a coaptation is worth aiming at is the amount of excursion required at the particular location of the nerve injury. When the tension is excessive, surgical mobilising of the nerve is required; the surgeon has to extend the extensile incisions proximally and distally to expose the nerve, mobilising the nerve from its surrounding soft tissue bed and allowing tension to be distributed over a greater length of the nerve itself. The surgeon can then ascertain the amount of tension required to hold the repaired nerve ends together and hopefully perform a tension-free primary nerve repair. However, the longer the delay, the more likely it is that grafting will be needed to bridge the gap, and the less likely it is to reach a successful outcome. An early repair is therefore always worth aiming for.

Degeneration of end organs

After a peripheral nerve injury with prolonged denervation, the end organs undergo several changes. The contractile response of the muscle fibres to impulses transported by the regenerating nerve axons diminishes (151). Muscle fibres atrophy quite rapidly with an initial weight loss of 30% in the first month after nerve lesion (40). In these cases, the affected muscles undergo microscopically degenerative processes including fibrosis (detectable in the muscle as early as 3 weeks post injury (152) and diminution of the motor endplates and the percentage of motor endplates with axon terminals (153). If the muscle is not soon reinnervated, fibrosis gradually progresses and will replace the muscle completely within approximately 2 years (154). Even when successful reinnervation has taken effect, the muscle will rarely be able to return to its' normal strength (155). Prolonged denervation with secondary changes in sensory end organs like atrophy in skin receptors etc. has been described in previous studies as well. Optimal recovery will occur when maximal numbers of regenerating nerve axons reach the correct target in the shortest time after injury, thus reducing the end-organ atrophy associated with denervation.

Considering that the distance between a proximal lesion and the muscle or sensory target can be substantial, adding the denervation time, and the fact that the rate of axonal regeneration, even in favourable circumstances, rarely exceeds 1 mm/day, the

reconstructive surgery should, without a doubt, be given priority and be performed in a timely fashion.

Although the examples given here are not all-inclusive, they do represent important aspects of mechanical and biological reasons why the primary repair of an injured peripheral nerve must be a priority. In clinical practice, end-to-end microsuturing of acute peripheral nerve injuries and autologous nerve grafting in severe nerve lesions, with nerve defects, will probably continue to be the standard method. In certain situations, however, technical and/or practical circumstances can cause microsuturing to be overly challenging. With an obvious need for additional methods, bonding techniques have been suggested as an alternative to sutures. These techniques should offer a rapid and reliable primary repair while achieving proper coaptation of the nerves. Cyanoacrylate adhesives (CAs) have been shown to satisfy these qualities in several aspects and have been tested in different clinical contexts as described above. The following is a summary of studies in which CAs have been used to repair peripheral nerve injuries.

Cyanoacrylate in nerve repair

Researchers in peripheral nerve repair have long recognised that sutures used in joining peripheral nerves strangulate the blood flow around segments of nerve fibres and also tend to distort the internal fascicular structure at the site of junction. Both of these factors interfere with the regeneration of axons. If possible, they should therefore be eliminated. Much effort has been made to deal with these deficiencies. During the 1940s and 1950s, several attempts were made to use plasma and fibrinogen products fortified with different extracts to repair transected peripheral nerves. None of these were found to be sufficiently successful to become an accepted and approved procedure. These failures were mainly due to the difficulties in the fashioning of the plasma. In addition, the strength of these bonds was inadequate to hold nerve ends together during the physiological healing process.

After the early 1960s, several new reports on research within this field were made public. This was especially noticeable after the introduction of synthetic adhesives: particularly of adhesives, which are relatively safe and compatible with biological tissues.

Short chain derivatives for peripheral nerve repair proved early on to be unsuitable. Ferlic and Goldner used methyl 2-cyanoacrylate to anastomose the cut ends of posterior tibial nerves in rabbits (156). The nerve ends were held together during the polymerisation period after a liquid adhesive had been dripped around the anastomosis. Approximately 1/3 of the studies showed some sign of unity. Unfortunately, they also showed signs of unsatisfactory axonal regeneration when the rabbits were killed at some time between the 28th and the 40th postoperative day. All of the repairs showed degeneration and necrosis. A general opinion was that nerve damage had been caused by heat, which was generated from the polymerisation process. On an experimental basis, Braun showed that alkyl 2-cyanoacrylate was associated with an adverse tissue reaction two and four weeks after peripheral nerve repair. Large and small mononuclear

cells routinely accumulated around the repair site (157). The tissue adhesive spread to the epineural surface around the readapted nerve endings. The inclusion of adhesive between the cut nerve ends was thought to prevent successful nerve repair.

During the early 1980s, Krysiak et al. injected butyl-cyanoacrylate (Histoacryl®) around a closed gap after transection of sciatic nerves in rats. Six months after the repair, they noted an electrophysiological recovery, similarly to that of sutures. However, morphological examination revealed a strong foreign body reaction around the repair site. There were degenerative changes with severe demyelination and axonal loss which could not be seen in the suture cases (158). Włodarczyk et al. injected butyl-cyanoacrylate (Histoacryl®) into and around a gap in an isolated and transected frog nerve. They then noted that electrical activity was obliterated (159). Today we know that after a nerve injury, both nerve endings will swell, without exception, in the early phase of regeneration. Since the adhesive collar is rigid, the local blood flow will be reduced when using CA in the repair seam. This will obviously influence the spread of nutrients and thus undermine the healing process as a result.

Furthermore, when a collar of CA is moulded around the readapted nerve endings, as in the cases described above, it is reasonable to assume that a large amount of adhesive was used in order to maintain contact between the nerve endings during the healing process. It is a known fact that the heat that is generated during CA polymerization is strongly influenced by the amount of adhesive used. This heat will potentially cause cellular damage and should be added to the cytotoxicity, which is proportional to the dose (160). The large amount of adhesive used is presumably one of several reasons for the unsatisfactory outcome in the previously described studies. This also appears to be a reason for the disappointing results in other studies where the amount of CA has obviously been too large in relation to what is mechanically necessary to maintain the seam during the healing phase process (83, 161).

In contrast to the above negative reports, Mielke reported favourably on the use of butyl-cyanoacrylate for grafting an interrupted recurrent nerve (162). Siedentop and Loewy looked at transected facial nerves in dogs, which, two months earlier, had been repaired with either butyl-cyanoacrylate or epineural sutures with a Silastic sheath wrapped around the repair site (163). The two methods produced equally good functional and morphological recovery. They concluded that synthetic adhesive was easier to use and less time-consuming.

In several studies within the last decade, researchers have begun to respond to the toxic properties of the adhesive and sought to minimise the amount used. The results from these studies have proved promising. Choe et al. showed neither discernible fibrous thickening surrounding the CA, nor any harmful effects on axonal growth across the anastomotic site when a minimum amount of CA was applied in between the overlapping of the epineuria of the two transected nerve endings (94). Pineros-Fernandez et al. demonstrated a new technique, by “painting” the adhesive in small amounts around the nerve. Results from this new method showed a faster return of nerve function quantified through walking-track analyses without indication of tissue toxicity when octyl 2-cyanoacrylate was compared to microsutures (164).

Ethyl-cyanoacrylate, our choice in nerve repair

In the search for an efficient biodegradable synthetic adhesive for repair of peripheral nerve injuries, we chose Evobond® for this thesis. Evobond® is a commercially available ethyl-cyanoacrylate (ECA) with a minimum of additives. The results from the experiments with rats, in *studies I, II, and IV*, were consistently compared to conventional microsuturing. Six months after lesion and repair of the sciatic nerve, the local tissue reactions in proximity to the seam indicated that ECA induces an increased inflammatory reaction, with accelerated Wallerian degeneration as a result. The redistribution of the α -motoneurons was scattered throughout a larger area and, regardless of which method had been used, the count was elevated within the spinal cord grey matter, while the synaptic coverage, compared to the controls, declined. In addition, the morphological and functional recovery was determined to be comparable to that of conventional sutures six months after repair.

In all experimental series the adhesive has been found to have satisfactory holding properties during the predetermined postoperative periods. The method did not show any sign of dehiscence among the sealed nerves throughout the studies. Since we published our first study, “Long-term results of peripheral nerve repair: A comparison of nerve anastomosis with ethyl-cyanoacrylate and epineural sutures”, in 2006, several other reports have been published with promising results concerning this topic (165-167). Rickett et al. used a guinea-pig model in their experiment to analyse the mechanical properties of transected sciatic nerves, repaired with epineural applications of ECA (167). They used a minimal quantity of the adhesive, which was thinly applied to the epineurium and thereby minimised seepage into the nerve core. The nerve, which was anastomosed with ECA, retained approximately 30% of an intact nerve load with preservation of nerve elasticity. The anastomosis failed when the load (0.93N) exceeded that of microsuturing, although not significantly so. The bond strength was assessed to be sufficient in order to withstand the subpathological *in vivo* forces, which occur during the healing period. These results correspond with our assumption that a very small amount of adhesive is sufficient, as long as it is applied accurately.

The bonding resistibility of CAs has, however, been debated, since the tensile strength has been proven to be inversely proportional to the cytotoxicity (97). The degree of harmful tissue reaction in the local area of application is influenced by the rate of elimination of adhesive, which depends on the rate of degradation combined with the vascularity of the surrounding tissues. When the alkyl chains of the molecular structure increases the amount of carbon, the compound degrades at a slower rate, resulting in less toxic levels of the by-products compared with derivatives of cyanoacrylate with short alkyl chains (168). The tensile strength is, however, generally less with longer chain derivatives (97). These conditions could militate against the appropriateness of using ECA in nerve repair since its alkyl chain is relatively short. However, there is another important factor that has to be considered: the viscosity of the liquid adhesive. CA homologues with longer alkyl chains have low viscosity and will therefore tend to contaminate the surrounding tissues when applied to non-horizontal surfaces. Histoacryl, currently the only CA that is approved for medical applications in Sweden, is a butyl-cyanoacrylate with a relatively long alkyl chain and, consequently, a CA with low viscosity. It is therefore difficult to handle this type of

adhesive without it spreading in the wound before hardening. This is of course particularly true within hard-to-reach areas. This problem could possibly be overcome if the glue could be encased during the polymerisation phase.

In two recently published studies, Histoacryl was used in a new application technique showing regeneration comparable with sutures 12 weeks after nerve repair (94, 165). This method seems promising in terms of containing the adhesive. By overlapping the epineuria of the two nerve ends and then applying a small amount of the adhesive to the surface of the epineurium, just where the epineuria overlapped each other, a reliable seam is suggested to be obtained. However, when both epineuria are stretched and overlap each other, the harmed axons are inevitably squeezed together. This, however, provides a smooth and attractive epineural surface, but it is achieved without the surgeon having control of the axonal structure inside the epineurium. This situation has similarities to nerve repair done with microsuturing. In *paper II* we were able to demonstrate that neurofilaments were poorly orientated in the middle of the repair site, giving the impression that the nerve endings had been forced together by pulling the epineurium together with sutures. On the other hand, when repaired with ECA, the entire cross-sectioned surface, both the proximal and the distal nerve endings, seemed to have been drawn together, as revealed by the alignment of the neurofilaments, and well structured into the interface of the adhesive. We concluded that this position of the previously transected axons could be favourable during the initial sprouting process. Moreover, CA homologues with shorter chains, such as ECA, have a high viscosity and do not spread as easily and form, instead, a droplet of liquid (168). These spreading characteristics allow precise application of the adhesive, regardless of the surface's orientation, which in turn would help to keep the wound from being contaminated. Hence, only a minimal amount of adhesive needs to be applied between the two nerve endings; just enough to form a solidified thin layer of liquid. Hereby, the cytotoxicity that is proportional to the dose used (160) and the cellular damage due to the exothermic reaction could be minimised

As discussed above, Schwann cells upregulate the expression of several neurotrophic factors when the axonal continuity is broken, in anticipation of the arrival of a regenerating sprout (143-145). This has been proven by analyses of fluid in the adjacent area of peripheral nerve repair, which has been shown to contain neurotrophic activity for motor and sensory neurons (169). This would suggest that neurotrophic support plays a physiological role in nerve regeneration. Initially we were concerned that the solidified thin layer of the synthetic adhesive between the two joined nerve endings would constitute an obstruction. Given that the initial healing process depends on a strict time schedule for how these trophic factors and other associated compounds interact during the initial regeneration, a compact layer between the joined endings might possibly hinder regeneration. However, in *paper II*, as soon as seven days after a delayed repair, neurofilaments were arranged in longitudinal bundles, amidst and around the adhesive, which was partially fragmented. The arrangement of neurofilament bundles in the proximal stumps, incapable of breaking through solid adhesive remnants, gave us the impression that the regeneration front searched its way through gateways in the adhesive seam. As bundles of neurofilaments appeared to cross the adhesive seam with relative ease, it is not difficult to imagine that molecular

structures like neurotrophic factors could diffuse, completely unhindered, from one side to another, in either direction in this area. We can only speculate with regard to whether these pores within the adhesive area were caused by phagocytosis, rapid biodegradation or cracks in the material due to micromovements. Neither the morphological nor the functional estimations lead us to believe that the adhesive seam could mechanically prevent the trophic factors from search out where they are most needed. However, whether or not the transient cytotoxic effect of ECA revealed in *paper III* has any harmful influence on the Schwann cells, and thereby on the neurotrophic support in the early phase of regeneration, remains to be explored in the future.

Future perspectives

Our results provide evidence that this method is beneficial and thus call for further studies using clinically relevant experimental models such as delayed nerve repair.

The long-term studies in this thesis have involved immediate repair only. In our *paper II*, in which we compared the outcomes of immediate and delayed repair, we observed some distinctions concerning the repair joints as early as after seven days of adhesive adaptation. Despite an increasing amount of information showing that delayed repair is inferior to immediate repair (43), the former is widely used for practical reasons in the clinical situation. It would be of considerable interest to compare the results obtained in our *studies I, II, and IV* with clinically relevant experimental studies in which delayed repair of the peripheral nerve was done. It seems likely, from a speculative point of view, that this course of action will prove to be fruitful in distinguishing the relative value of immediate and late repair of peripheral nerves.

In our *paper III*, we compared ECA with a CA approved for medical use in an *in vitro* cell culture trial. Both adhesives show evidence of transient cytotoxicity. In the light of previous experimental studies and in this *in vitro* cell culture study, we found that there is no toxicological evidence indicating that ECA could not be used instead of BCA in peripheral nerve repair when a synthetic tissue adhesive is preferable. However, further investigations into the toxicity of ECA to human nerve cells is necessary to find out whether it may be useful in clinical trials as a possible option to microsutures in the repair of peripheral nerves.

Unfortunately, many nerve injuries do not undergo primary repair. This is particularly prevalent in situations associated with extensive traumatic injuries as in natural disasters, in large-scale (traffic) accidents, during times of war or for people suffering from relatively mild trauma, including nerve lesions, in parts of the world where the material resources are markedly limited. If this complementary surgical technique, simple and inexpensive, could fully prove to be a safe method for the repair of peripheral nerve injuries, many domestic patients and, above all, patients in large parts of the world where the material resources are more scarce could be treated for traumatic injuries that would otherwise probably result in prolonged and unnecessary suffering.

Beyond the horizon...

Some experimental reports can be found in which fibrin sealants containing neurotrophic factors have been used in conjunction with sutures in order to repair injured peripheral nerves (170). The fibrin sealant had then previously been enriched with a certain combination of trophic factors. These were gradually released in the repair area (171). NGF and NT-3, as well as GDNF, have been shown to enhance nerve regeneration to various degrees. This is explained by sustained local delivery of trophic factors to the damaged nerves.

Furthermore, there are new molecular biology techniques in which specific genes are inserted into neuronal cells with the hopeful expectation that they will produce their own “trophic support molecules” (172). As a result, some have proposed the construction of nerve grafts of cells which have been genetically manipulated to produce growth factors (147). These two options, involving external supplying of trophic support, could certainly be successful, but it would be more desirable to create a safe nerve repair tool combined with a “built-in function” of sequential and sustained release of trophic factors.

The idea of using nanospheres or nanopowders in biomedical applications as drug carriers has been discussed since the introduction of nanotechnology in the 1980s (173). A nanosphere is a microscopic hollow sphere of cyanoacrylate polymer that can be filled with either an active substance or with the active substance adsorbed on its surface (174). In a future perspective, these nanoparticles could perhaps be produced from CA with different lengths of the alkyl chain, loaded with appropriate neurotrophic factors, and compounded with ECA. The trophic support around the adhesive-repaired nerve endings could hereby be controlled in a sequential and sustained release manner and probably optimise the possibilities of nerve regeneration.

CONCLUSIONS

- The use of ethyl-cyanoacrylate adhesive for anastomosing transected peripheral nerves gives adequate tensile strength during regeneration;
- Anastomosis of a transected peripheral nerve with ethyl-cyanoacrylate adhesive supports morphological and functional recovery comparable to that of microsutures;
- The use of ethyl-cyanoacrylate adhesive for anastomosing transected peripheral nerves induces an increased inflammatory reaction in the area around the repair site, which may lead to accelerated Wallerian degeneration and could therefore have benefits over conventional microsutures for the reconstruction of peripheral nerves;
- The polymerised thin layer of synthetic adhesive between the two joined nerve endings becomes partially fragmentised during the first week after repair and allows regenerating axons to cross the seam;
- Using an adhesive to close a gap between nerve endings by drawing the entire cross-section area of the proximal and distal nerve endings together results in a well-structured alignment of the neurofilaments in a more anatomical manner compared to microsutures;
- There is no toxicological evidence indicating that ECA could not be used instead of BCA in peripheral nerve repair when a synthetic tissue adhesive is preferable;
- The selectivity of motor reinnervation following sciatic nerve transection and subsequent repair with ECA is comparable to that following conventional microsuturing.

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