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PROCESSES OF PERIPHERAL NERVE AND NEUROMUSCULAR REPAIR

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Summary. - *The purpose of this review is to describe the most recent, and debated, avenues of investigation relevant to the processes of nerve regeneration. Special emphasis is placed on: 1) the role of Schwann cells, macrophages, and basal laminae during Wallerian degeneration and reinnervation; 2) the possibility of achieving a synchronization in regeneration rate of different axonal types; 3) the choices that regenerating axons make when they reinnervate muscle; 4) the sequence of events which leads regrowing nerve fibers to reach and reconnect functionally with their peripheral targets; 5) the soluble, membrane-bound, and extracellular matrix molecules that may guide axonal behavior; and 6) the agents which may be effective in modulating the functional and structural recovery of damaged peripheral nerves. It is concluded that, in order to achieve a perfect repair, a) the regenerating neurites must find a continuous, unbroken chemico-physical pathway to appropriate targets through a supportive milieu, and b) any effective pharmacological manipulation must ultimately act on bridging gaps in fiber pathways by minimizing the disruption of support elements and/or maximizing the delivery of growth-stimulating substances to the lesion site.*

Riassunto (Processi di riparazione neuromuscolare e dei nervi periferici). - *Il proposito di questa rassegna è di descrivere i più recenti e dibattuti approcci della ricerca riguardante i processi della rigenerazione nervosa. Un accento particolare è posto su: 1) il ruolo delle cellule di Schwann, i macrofagi e le lamine basali durante la degenerazione Walleriana e la reinnervazione; 2) la possibilità di raggiungere una sincronizzazione nella velocità di rigenerazione di assoni di tipo diverso; 3) le scelte che gli assoni rigenerantesi compiono quando essi reinnervano il muscolo; 4) la sequenza di eventi che porta la ricrescita delle fibre nervose a raggiungere e riconnettersi funzionalmente con i loro bersagli periferici; 5) sia le molecole solubili, sia quelle legate alle membrane e quelle della matrice extracellulare che possono guidare il comportamento assonale; 6) gli agenti che possono essere efficaci nel modulare il recupero strutturale e funzionale dei nervi periferici danneggiati. Si è concluso che per poter raggiungere una riparazione perfetta: a) i neuriti rigenerantesi devono trovare un percorso chimico-fisico continuo, non interrotto fino al raggiungimento della meta attraverso un ambiente di supporto, e b) qualsiasi manipolazione farmacologica efficace deve agire soprattutto a colmare la interruzione nei percorsi delle fibre attraverso la minimizzazione della distruzione degli elementi di supporto e/o massimizzando il trasporto di sostanze stimolanti la crescita fino ai punti dove è avvenuta la lesione.*

Introduction

Generally speaking, when any axon from either peripheral nervous system (PNS) or central nervous system (CNS) is severed, part of the neuronal cytoplasm becomes isolated from the part of the nerve cell containing the nucleus and most of the organelles necessary for the synthesis of structural proteins. The detached axonal piece, together with the associated myelin, if any, degenerates following a fairly stereotyped sequence of events known under the general name of Wallerian degeneration (see further). While the distal axonal fragments are eventually phagocytized, the surviving proximal stump may produce a new length of axon to replace that lost at the time of injury. It has been observed that all types of axons (myelinated, unmyelinated, motor, sensory, autonomic) can regenerate effectively, though within certain limits, and that success or failure in reinnervating the structures they formerly supplied is determined largely by the anatomical site at which the nerve fibers are transected.

The present paper will review briefly the mechanisms leading to regrowth of amputated axons as well as the most recent data about identified or putative processes able to modulate axonal regeneration. This article will focus particularly on topics which are currently under active investigation because of their significance and/or interpretation difficulties. Moreover, only phenomena taking place within the territory of the peripheral nerv-

ous system will be considered, since the limited capability of CNS neurons to regenerate in adult vertebrates is still a controversial matter and has been already dealt with in a number of recent reviews [1, 2].

Wallerian degeneration and the role of Schwann cells

A considerable body of experimental data has emphasized that peripheral nerves in both humans and other mammals display a characteristic and reproducible degeneration following any kind of injury, whether by transection, crush, severe compression or by other means [3].

The outlines of degenerative changes taking place in nerve fibers distal to a nerve cut had been already described by Waller in 1850 [4] and since then termed collectively as "Wallerian degeneration". Since these changes may either lead to axonal regeneration or alternatively end in neuronal death, they have been one of the most intensively studied topics in neurobiology. It is known that following nerve transection, Wallerian degeneration is limited to distal portions of axons which have been separated from the biosynthetic apparatus of the nerve cell cytoplasm, as well as to the immediate neighborhood of the lesion in proximal axonal portions. The remaining proximal axons, their somas, ascending axons and other processes undergo both functional and morphological alterations referred to as the "axon reaction" or the "retrograde effect" [5]. These retrograde effects include morphologically: fiber atrophy and/or loss, decreased cytoplasmic basophilia (the classical "chromatolysis" [6]), and structural alterations of the nucleus. Retrograde effects are more severe when: 1) the nerve is cut rather than crushed [7]; 2) regeneration to target tissues is prevented instead of facilitated by reconnecting cut ends [8]; 3) young rather than mature animals are studied [9, 10]; 4) the transection is nearer the cell body [11]; 5) sensory as opposed to motor fibers are involved [12]; 6) observation times after amputation are increased [13]. Finally, the effects may vary with the nerve and with species [5].

It is relevant to the aims of present review to note that the mechanisms underlying the initiation of the "axon reaction" to distal injury are not yet fully understood. Several possible "triggering" signals have been proposed in the past, including depolarization of the membrane, loss of axoplasm, lack of substances usually conveyed by retrograde axonal flow, and/or occurrence of a substance originating at the site of injury and conveyed to the cell body by retrograde axonal transport [14]. While the possible role of peripherally-derived neurotrophic factors acting in a retrograde manner on spinal motoneurons will be discussed in a further section, it is worth reporting here that blockade of retrograde axonal transport by colchicine applied proximal to the site of nerve transection delays the onset of metebolic and morphologic changes induced by axotomy [15].

By concentrating now on the phenomenon of Wallerian degeneration, it can be observed that, like for the "retrograde effects", the time-course of appearance of degenerative changes in the peripheral stump is also influenced by the topographical level of nerve transection [16]; and pattern and spatial evolution of the changes are less severe following crush than after nerve ligation or severance [17]. The sequence of events forming the Wallerian degeneration as a whole may be divided into primary effects, such as accumulation of organelles in paranodal axoplasm [18] and loss of axoplasmic microtubules and neurofilaments [19], and secondary effects involving myelin and Schwann cells. In the latter category, the first visible step of incipient degeneration is the activation of hitherto relatively quiescent Schwann cells. This event occurs as early as 12 hours, accompanied in the following 3 days by an increase in the number of Schmidt-Lanterman incisures, a loosening of the myelin lamellae, and retraction of myelin loops at the nodes of Ranvier [20]. In the remaining first week of degeneration, segmentation of myelin appears paranodally, and myelin debris are taken up by cells which resemble activated Schwann cells [21]. However, recent research on the role and identity of cells responsible for the digestion of myelin during Wallerian degeneration has shown that Schwann cells neither proliferate nor phagocytize the myelin [22]. Since there is no resident population of phagocytes in the nerves, the increase in cell density and myelin phagocytosis is entirely dependent on the entrance into the nerve of non-resident cells, which appear to form a subset of hematogenous monocytes carrying Fc receptors on their surface [23]. These observations have been confirmed *in vitro* on explant cultures of cat sciatic nerve which do not contain macrophages [24]: thus Schwann cell proliferation in Wallerian degeneration is directed towards re-establishing cellular continuity within the basal lamina tube which is lost when Schwann cells reject their "old" myelin sheaths.

According to these recent findings, a new interpretation may be offered as regards the early responses of Schwann cells to nerve injury (cytoplasm hypertrophy, increase in organelles and cytoplasmic movements, invasion of the axon by the hypertrophied adaxonal layer [16], loss of micropinocytotic vesicles and other membrane changes [25]). In the so-called "peritraumatic zone" [18] the nucleus of each activated Schwann cell comes to be sited between portions of disrupted myelin sheath, while other Schwann cells of myelinated fibers never seem to reach the mitosis status and remain sited adjacent to a myelin sheath surrounding the axon. Thus it is possible to distinguish between premitotic Schwann cells, associated with the myelin of degenerating nerve fibers, and mitotic Schwann cells, the nucleus of which assumes a more central position in the axonal cross-section, bisecting both the myelin sheath and the axon before or during division [26]. This appears to be the explanation for the "hypertrophy" of juxtanuclear cytoplasm and indentation of the myelin sheath that precede ovoid formation in nerves undergoing Wallerian degeneration [16].

The behavior of Schwann cells in the early weeks following nerve injury is crucial to success or failure of ensuing possible regeneration. From the data summarized above it can be drawn that: 1) myelin debris are probably not necessary for the stimulation of Schwann cell mitosis in degeneration; 2) the signal activating Schwann cells to divide originates at the site of injury and spreads both centripetally to determine the typical "retrograde effect" ("axon reaction") and centrifugally along the affected portion of nerve at rates that are consistent with those of fast axonal transport; 3) the stimulus for Schwann cell mitosis during nerve regeneration might be either the loss of an axolemmal component or the appearance of an axolemmal protein-associated mitogenic signal specific for Schwann cells; 4) separation from axonal contact may be the basic factor in determining which Schwann cell population will eventually proliferate, migrate, serve as guidance for axonal sprouts, and form new myelin sheath.

Investigations are currently focusing on materials that Schwann cells in the distal segment of transected nerve secrete at different stages of degeneration and regeneration. Nature, amount and specificity of the secretion appear to play an important role in the promotion of axonal regrowth, in the remyelination of surviving sprouts, and in the processes leading to the reconnection with denervated targets. If, for instance, axons are prevented from reentering the distal nerve segment, Schwann cells are forced to pass from a myelin-forming or myelin-maintaining to a quiescent state characterized by a down-regulation in the biosynthesis of the mayor myelin glycoprotein, P_0 [27]. Likewise, pretreatment of injured nerves with neurotoxic substances [28] or X-irradiation [29] impairs the regenerative capability of the nerves, probably due to inhibition of the secretion of neurotrophic substances by the Schwann cells. Among these putative substances, it is worth recalling here a heparan sulphate proteoglycan associated to the basal lamina [30] which seems to have a crucial role in restoring the contact between axonal tips as well as their accompanying Schwann cells and the target muscle fibers [31, 32]; and the apolipoprotein E, a 37 kDa protein which is synthetized in response to nerve injury to mobilize lipids produced as a consequence of axon degeneration [33] and which might be involved in the promotion af axonal regrowth by participating in lipid reutilization, especially redistribution of cholesterol to neurons needing this lipid for axon growth and to Schwann cells for remyelination [34-36].

Conditioning lesions

Severed axons regenerate more rapidly than usual after a second ("test") lesion if they have been injured by an earlier ("conditioning") lesion. On the basis of what has been summarized above as regards the Wallerian degeneration and the retrograde axonal reaction, it could seem that a "priming" in a distal part of the axons would shorten the delay period necessary for the changes in molecular mechanisms involved in nerve regeneration to appear [37]. Actually, a more vigorous metabolic response to the conditioning lesion and an enhanced shift in protein synthetic activity from functional proteins (associated for instance with synaptic transmitter metabolism) to structural proteins (cytoskeletal proteins, such as tubulin, actin, and neurofilament proteins) have been proposed by several authors, but never fully demonstrated [38]. On the other hand, studies on the time course of the conditioning lesion effect on axonal regeneration have shown that an accelerated outgrowth can be achieved either by conditioning intervals as short as two days or as long as 28 days [39]. These figures are inconsistent with times usually necessary to the nerve cell body to respond maximally to axotomy [40] and cannot be related exactly either with fast or slow retrograde axonal transport of regulatory substances.

In order to explain at least some of the features characterizing the conditioning lesions, several possible mechanisms have been proposed. First of all, it has been noticed that the rate at which axons regenerate after being damaged (2 to about 6 mm/day) is comparable in most nerves (and in several animal species as well) with the rate of anterograde axonal transport of proteins forming the bulk of so-called slow component b (SCb) of axoplasmic flow, i.e. actin and tubulins [41]. However, it has also been observed that both capacity for regeneration and locomotion speed of the axonal growth cones differ among nerve fibers of similar histologic appearance. For instance, a number of authors has reported that, in motor nerves, reinnervation of muscle by fusimotor γ fibers requires much longer than reinnervation by large-sized α motor axons [42, 43]; likewise, the skeletofusimotor system (β axons), in which intrafusal and extrafusal muscle fibers share a common innervation, and which displays a different localization within any muscle spindle as opposite to the intrafusal γ system [44], follows regeneration patterns which may be markedly at variance with those of other motor fibers forming the innervation of either spindles or ordinary ("extrafusal") muscle fibers [45]. It is therefore conceivable that a conditioning lesion may enhance regeneration speed and efficiency just by synchronizing the regeneration rates of individual motor axon populations in a single nerve.

This view is indirectly supported by the observation that sensory fibers as well show regeneration patterns which mirror the differences detectable among nerve fibers in the course of degeneration [46, 47]; in particular, following transection, crush and nerve freezing, degeneration in several types of sensory axons tends to take longer than that occurring in any kind of motor fiber. Additionally, in some structures, such as neuromuscular receptor organs [48] and cutaneous mechanoreceptors [49], degeneration may be not followed by regeneration or it might take years before all afferents have regained their original caliber, functional properties and maturative features. Since it has been shown that small-diame-

ter sensory axons are under-represented in samples of successfully regenerated fibers [46] and that in unmyelinated sensory axons the regeneration speed does not necessarily correlate with the rate of axonal transport of SCb [50], it may be postulated again that in mixed nerves, such as the sciatic, the favorable effect of conditioning lesions on axonal regeneration could be dependent on a specific acceleration in the sprouting of those fibers which usually grow out at later times or slower rates; in such a way, the bulk of regenerating fibers is upleveled to travel to reconnect with peripheral targets at the highest possible speed compatibile with capacity of the synthetic machinery of the cell body and maximal speed of cytoskeletal axonal transport. A beneficial action specifically on slowest-moving axons may in turn be due to special messages from or differences in the target cells to be reinnervated [51].

Sprouting, collateral reinnervation and direct nerve regeneration

When muscles are denervated by severing their nerve supply, there are two ways by which they may become reinnervated. These are, first by sprouting and ingrowth of intact nerves from adjacent muscles (so-called "collateral reinnervation"), and secondly by the return of regenerating sectioned nerve fibers. Collateral reinnervation may begin within 4 days of denervation [52], but the return of regenerating fibers ("direct nerve regeneration") may take a longer and variable time depending on the distance over which the damaged axons have to regenerate.

Under normal conditions (i.e. without any specific treatment, which will be dealt with in a further section), regeneration of a lesioned nerve is featured by early entrance of axons into the denervated muscles, appearance of subthreshold endplate potentials (e.p.p.s.), and formation of new branches which ultimately make multiple synapses on a single endplate or on the same muscle fiber ("polyneuronal innervation") [53]. Such a pattern is reminiscent of fetal maturative processes in the nerve-muscle relationships, and includes the ensuing gradual reduction in polyinnervation, which helps restoring the ratio nerve fibers/targets up to reaching the right number of functional motor units [54].

While the mechanisms by which axons from neighboring nerves can sprout out have been extensively described in the past and will not be repeated here, the fate of collateral sprouts after the "direct" regenerating fibers have returned to the denervated muscle remains to be fully elucidated [52]. Conflicting results have been obtained in this regard, according to which collateral sprouts are withdrawn, or remain but are functionally repressed, or remain as efficient as the "direct" regenerating fibers. The most likely explanation for these widely varying reports lies in the timing: because of their capacity of regaining normal density and properties, early-returning regenerating fibers will displace collateral sprouts; on the contrary, a late return will not determine any withdrawal of collateral sprouts [55].

Another point of controversial reports as well as of particular interest is the occurrence of sprouting and synapse formation following contralateral axotomy. Especially in frogs [56, 57], but to a lesser extent probably in some mammals as well, the intact nerve to the contralateral muscle sprouts and forms additional synaptic connections with already innervated muscle fibers. It has been assumed that the signal for nerve sprouting originates from the damaged (denervated) muscle cell and is transferred transneuronally to the contralateral side. Previous investigations from this laboratory have actually shown that after 30 days post-crush, the nerve supplying the rat extensor digitorum longus (EDL) muscle contralateral to the site of lesion presents several nodal sproutings (Fig. 1) which may account at least partially for the supernumerary innervation detected electrophysiologycally as multiple e.p.p.s.. However, since nodal sprouting is just one of the three main types of sprouting observed in both normal and injured muscles, it is questionable whether new branches visible in the nerve are really induced by the contralateral EDL motor nerve lesion or are they only the epiphenomenon of the continuous remodelling taking place at the nerve-muscle junction [58]. Figures 2 and 3, featuring the different extent and typology of sprouting in nerve fibers within soleus muscles of either side following unilateral resection of L_5 spinal root, emphasize that in the ipsilateral (lesioned) side all sprouting types are present (nodal, originating from the nodes of Ranvier; preterminal, originating from the unmyelinated preterminal segment of the axons before onset of terminal branching; ultraterminal, outgrowing from the terminal tree and extending beyond the endplate perimeter), whereas in the contralateral (intact) side sprouting is exclusively preterminal.

These unexplained differential responses may reflect intrinsic differences in specific sprout-inducing signals conveyed along intact vs. injured nerve fibers, such as trophic factors produced by the muscle (see further). Accordingly, sprouting of motor nerve terminals has been reported to be more vigorous and to occur sooner in slow-twitch muscles than in fast-twitch muscles, probably due to dissimilarities in both motor neuron and muscle fiber properties, as evidenced by electrophysiological, histoenzymological, and biochemical characteristics [59]. Studies aimed at finding whether sprouting and regrowth of axons occur at random or are dependent on some factor related to motor unit metabolic activities and organization [60-62] have pointed out that, following misdirection of regenerating axons to foreign muscles or cross-union of nerves supplying antagonistic muscles, several mechanical properties, such as tetanic tension, are redistributed in reinnervated muscles irrespective of the source of the regenerating nerves. This supports the view that inappropriate peripheral connections, while do not substantially modify the activity of spinal motoneurons, may determine a reorganization of

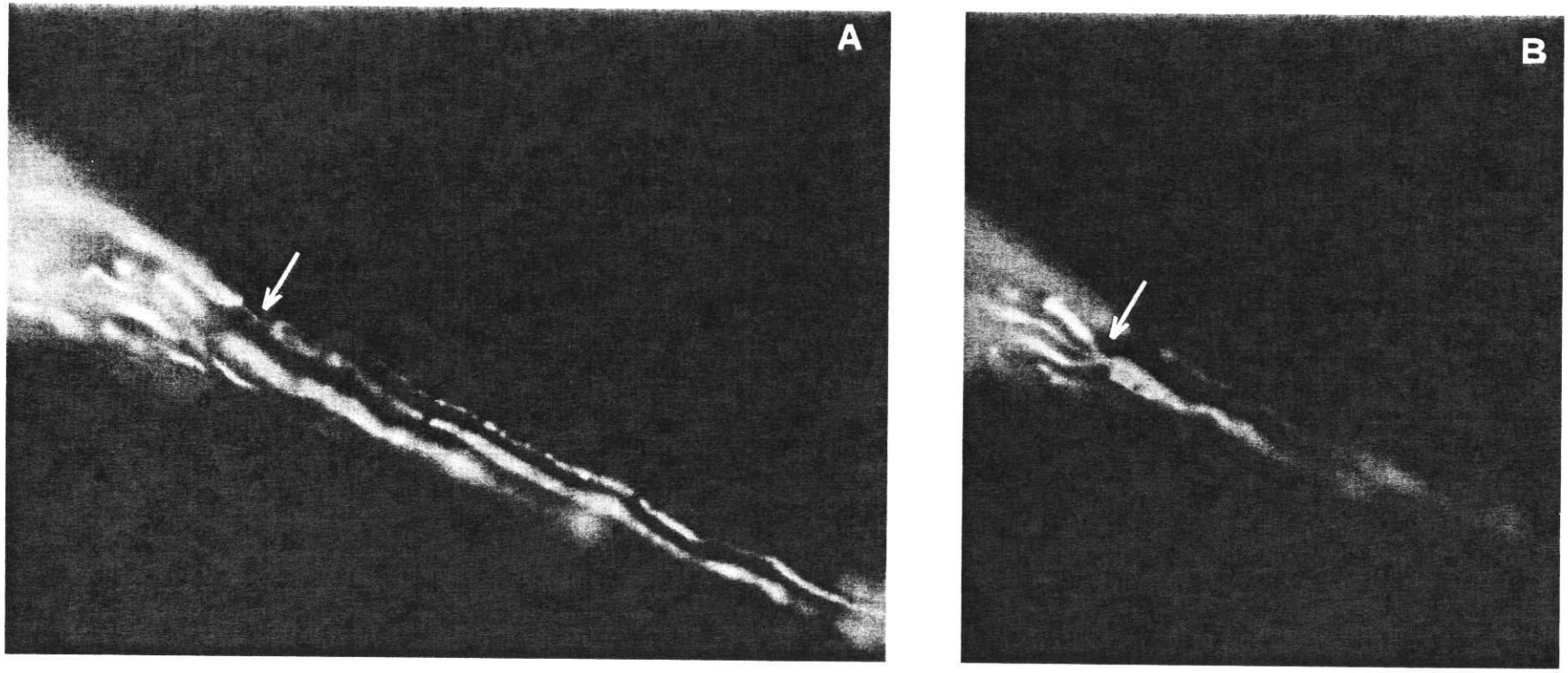

Fig. 1. - Intact nerve branch supplying the *extensor digitorum longus* (EDL) muscle in a rat, 30 days after a crush in the contralateral EDL nerve. Arrows in A and B point to two adjacent nerve fibers from which two (A) and three (B) nodal sprouts originate, respectively. These as well as the following micrographs were obtained from specimens stained immunohistochemically with fluorescein isothiocyanate-linked monoclonal antibody against the 150 kDa subunit of the neurofilament proteins. (120 x before photographic enlargement; final magnification: 600 x).

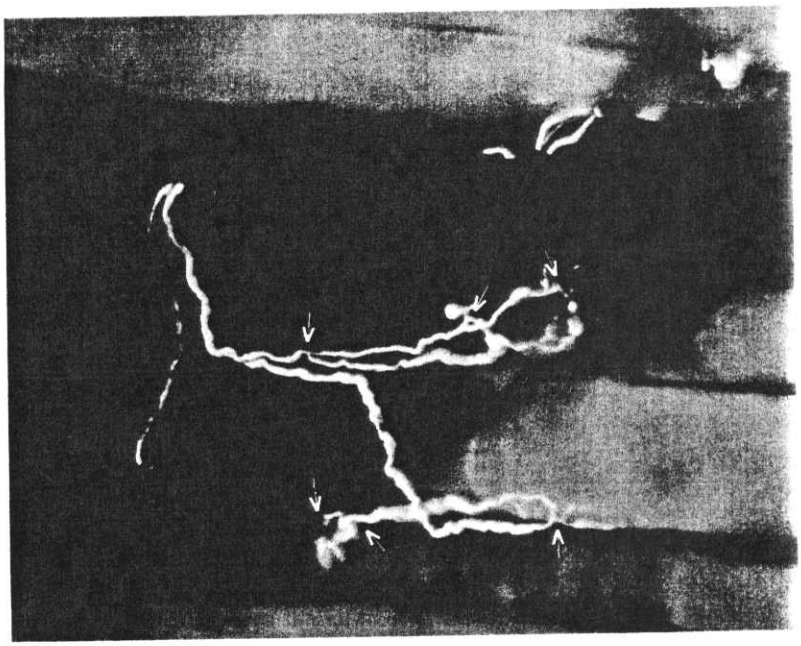

Fig. 2. - Regenerating nerve fibers in the soleus muscle 30 days following resection of the ipsilateral L_5 ventral root. Nodal, preterminal and ultraterminal sproutings are clearly visible (arrows). (80 x before photographic enlargement).

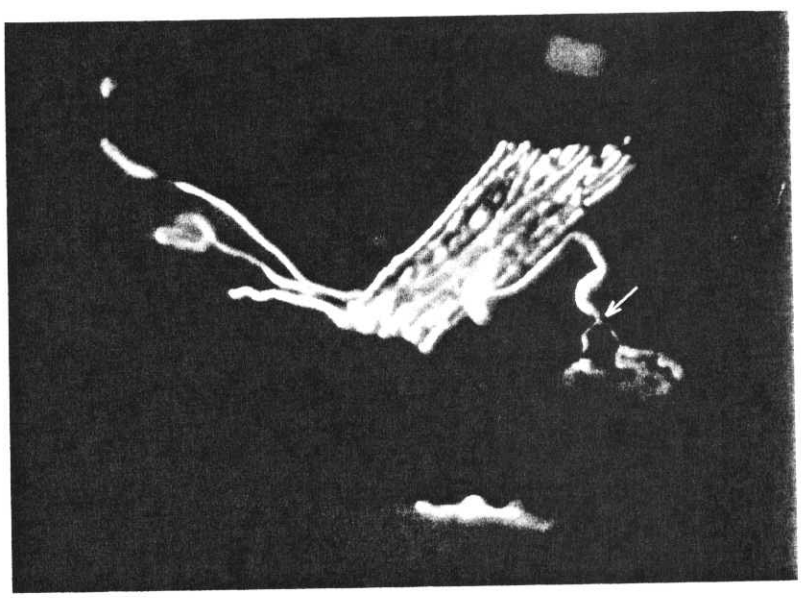

Fig. 3. - A bundle of nerve fibers innervating the soleus muscle contralateral to the root-resection site. The arrow points to a preterminal sprout making a double contact with a single endplate. (80 x before photographic enlargement).

nerve and muscle characteristics which do not obey the motor-unit size principle; since nerve fibers show no apparent preference for their former muscle fibers, recruitment of motoneurons in a motor pool might or might not elicit muscle contraction, depending on whether their regenerated motor axons reinnervated muscle at all.

Muscle-derived trophic factors and nerve-muscle relationships

If a muscle and its nerve are damaged in a way that spares the basal laminae of both muscle fibers and axons, the regenerating axons grow to reach the original synaptic sites on the muscle fiber basal lamina even in the absence of any surviving muscle fiber therein [63]. However, although the regenerating axon terminals differentiate normally, with accumulation of synaptic vesicles and formation of active zones, nearly all these maturative characteristics disappear from the synaptic sites within a few months. If, on the contrary, damaged axons are allowed to reinnervate intact muscle fibers for 1-2 weeks and then the muscle fibers are removed from their basal lamina sheaths, the differentiated axon terminals persist at the synaptic sites for at least 5 months. Such an axonal behavior is proper to somatic and autonomic motoneurons of vertebrates, since it has been repeatedly demonstrated that invertebrate nerve cells need not to contact their target muscle in order to survive and differentiate to maturity [64].

One possible explanation for these findings is that muscle fibers provide, in higher animals, factors that stabilize the axon terminal at synaptic sites. The existence of skeletal muscle-derived neurotrophic factors acting in a retrograde fashion on motoneurons had been postulated several years ago, on the evidence that muscle activity by electrophysiological stimulation was crucial to maintenance of motoneuron properties [65]. Additional pieces of evidence came from investigations on stimuli for sprouting (see above). It was found that terminal sprouting is stimulated in the absence of axonal degeneration when muscle fibers are rendered inactive by blockade of axon conduction or synaptic transmission [66]. Since muscle fibers are known to undergo profound changes in their properties when they are denervated or otherwise inactivated, it was proposed that a change in muscle toward a denervation-like status includes also the production of a stimulus for the motor nerve to grow out and to reconnect with its inactivated target.

The properties developed by muscle after denervation are similar to the properties of embryonic muscle (myotubes) at the time innervation is established: denervated and embryonic muscle fibers have, for example, acetylcholine receptors over their entire surfaces, and both will accept new innervations [67, 68]. The production of a motor nerve growth stimulus by denervated muscle (or by muscle in a denervation-like status) is therefore likely to be another example of reversion of the muscle to the embryonic status, when motor nerve are physiologically stimulated to branch and innervate the newly formed fibers. In support of this hypothesis it was reported that direct electrical stimulation of inactive or denervated muscle fibers inhibits both the denervation-like changes and terminal sprouting, whereas sprouting is stimulated in normally innervated active fibers in which denervation-like changes have been induced by mimicking nerve degeneration through inflammatory effects on the muscle itself [69, 70]. Likewise, in avian and mammalian embryos, procedures which reduce or block muscle function *in vivo* subsequently prevent the death of many motoneurons, delay the reduction of polyneuronal innervation, and induce motoneuron neurite sprouting [71]. On the contrary, electrical stimulation of such denervated skeletal muscle blocks the sprouting in neurons and increases the rate of loss of polynnervation [54].

Because denervation-like changes include alterations in the muscle fiber surface membrane, it has been suggested that these altered surface properties are the stimulus for terminal sprouting [72]. As discussed in the first section of the present review, another potential source of growth factors is apparently the Schwann cells that, in the denervated intramuscular nerves, tend to remyelinate the motor axons; whether the 37 kDa protein is the factor which act on the axon membrane at the nodes of Ranvier is still unknown, as it is a controversial matter why axonal growth is elicited only at peripheral nodes [73]. Actually, it has been observed that in partly denervated muscle, only nodes of Ranvier close to the denervated terminal branches of motor axons develop sprouts; by analogy, in muscles inactivated with botulinum toxin, nodal sprouts develop mostly at the last node of Ranvier before the nerve terminal [74, 75].

This localization of nodal sprouts to the peripheral branches of motor axons suggests that the stimulus for nodal sprouting, as for terminal sprouting, arises from the denervated or inactive muscle. However, nodal sprouting, unlike terminal sprouting, is not prevented by chronic direct electrical stimulation of the muscle. It follows that if the nodal sprouting stimulus is the same agent as the terminal sprouting stimulus, then it must be released from a site that is not affected by direct stimulation. One possibility is that it is released from the denervated endplate, whereas the terminal sprouting stimulus might be released from the extrajunctional region of the muscle fiber [76].

Extraction, purification, biochemical assay, and pharmacological characterization of some protein factors derived from rat hindlimb skeletal muscle after 5 days of denervation have been recently achieved [77]. One such factor displays neurite-inducing activity which is morphologically detectable as promotion of neurite elongation in cultured rat ventral spinal neurons; this factor is an acidic glycoprotein with an M_r of 35,000, i.e. within the weight range of the apolipoprotein E described above. It is worth reporting here that a saline extract of crushed adult muscle of rat was recently

shown being effective in stimulating muscle growth, myoblast fusion *in vivo*, satellite cell proliferation, and finally muscle regeneration [78]. The molecular weight of this factor as well appears to be greater then 30 kDa, probably nearer the 37 kDa mark.

The observation that atrophy in experimentally inactivated muscles is less than in denervated muscles, led to the assumption that many changes in denervated muscles are not due solely to the disuse caused by paralysis, yet the additional atrophy may be a consequence of the loss of neurotrophic substances normally delivered by intact motoneurons [79]. By injecting nerve extracts into mice, postdenervation physiologic changes in tetanus tension, time-to-peak twitch tension, and half-relaxation time resulted ameliorated. Nerve extract was also able to prevent completely the loss of parvalbumin in the denervated muscle and to promote morphological maturation, differentiation, and maintenance of aneural chicken muscle cells grown in tissue culture [80]. The factor responsible for these trophic effects was purified from chicken sciatic nerve, identified as an acidic glycoprotein, and referred to as "sciatin". It was later found that "sciatin" is the same as transferrin, an iron-binding glycoprotein present in serum and egg white; however, removal of transferrin from crude nerve extract did not diminish the ameliorative effects on denervated rat muscle [81]. Attempts at isolating and characterizing nerve-derived trophic substances have been only partially successful so far. It is possible that the most active factor is a glycoprotein with an M_r of approximately 100,000 [82].

Agents regulating and enhancing neuromuscular repair

The nerve growth factor (NGF) is the prototype of factors promoting axonal growth and regrowth [83]. A large body of evidence has cumulated in the past twenty years about NGF numerous effects, ranging from stimulation of neurite outgrowth from sympathetic and embryonic sensory axons, to promotion of neuronal phenotypic expression of immature adrenal chromaffin cells, prevention of embryonic cell death, and guidance of growing neurites. However, although most of the well-documented effects of NGF occur in peripheral systems, the motor neurons and their nerve fibers, i.e. the primary targets in many regeneration studies, are probably unaffected by NGF [84]. Recent observations from different laboratories have nevertheless pointed out that, in the course of regeneration following different forms of sciatic nerve injury (crush, permanent transection, desheathment), receptors to β-NGF are induced on the distal part of the nerve [85]. While the reinnervation after crush leads to a gradual decrease in specific receptor binding, which disappears after 25 days, permanent denervation prevents any repression of β-NGF receptors on the distal nerve. Whether these receptors may mediate any particular effect of NGF on motor axons or be the points from which NGF is selectively taken up to be transported retrogradely to motoneuron perikarya for exerting subsequently intracellular action, it remains an open question.

An interesting group of molecules which is currently under active investigation are the so-called neural cell adhesion molecules (N-CAM), a family of cell surface glycoproteins which share functionally important carbohydrate structure [86]. The glycoproteins that belong to this family are the neural cell adhesion molecules L1, J1, N-CAM, and the myelin-associated glycoprotein (MAG). It has been suggested that N-CAM plays a key role in binding of neurons to striated muscle cells, but structure-function relationships of individual neural cell adhesion molecules have been relatively little investigated in the PNS, especially as far as their roles during development and regeneration and under pathological conditions are concerned. Very recent studies [87, 88] have indicated that after denervation (by crushing or cutting the sciatic nerve): 1) the amount of N-CAM increases in the area surrounding the lesion; 2) L1 and N-CAM are detectable in neurites, Schwann cells, and the perineurium of the regenerating nerve; 3) the reduction of L1 expression to its normal adult level may take even one year, thus recapitulating normal development but on a more protracted time scale; 4) the amount of N-CAM in muscle fibers increases transiently on the surface and in the cytoplasm, and in interstitial spaces between fibers; 5) restoration of normal N-CAM levels in muscle is dependent on reinnervation: in a chronically denervated state, N-CAM levels remain high. Taken together, these data suggest that neural cell adhesion molecules play a number of roles in regenerative events and may have practical application in the clinical problems relevant to nerve repair, particularly in view of the function of these molecules in mediating cell-cell adhesion leading to tissue structures.

It is worth recalling here that in the past 20 years attempts have also been made to electrostimulate several sensorimotor as well as sympathetic nerves in order to enhance reparative processes and/or to avoid functional wasting of muscles paralyzed following spinal or supraspinal lesions. Many questions have arisen about type of stimulation (whether applied directly to the injury site on exposed nerve trunk, or by transcutaneous application), mode of supply (battery implants, direct or alternate current, caliber and material of electrodes, etc.), and parameters for evaluating nerve regeneration and motor function recovery. A matter of particular concern is the possible occurrence of electrolytic processes at the electrode-tissue interface due to long-lasting influence of low direct current. Although focal demyelination, axon losses, perineurial thickening and subsequent fibrous scarring have been reported, it is believed that these histological alterations could be the consequence of mechanical irritation produced by dislocation of electrodes [89, 90] rather than a side effect of current delivery.

Whereas these issues, although interesting, remain not directly related to the problem of regeneration, electrical stimulation has been used mainly to exploit elec-

tric currents that are generated at axonal membrane level for neurite development and orientation. Unlike the short-lived currents that occur with neuronal firing, these currents last hours or even days. Early studies in this connection have demonstrated that neurite outgrowth is accelerated and orients toward the negative pole or cathode of an electric field both in chick dorsal root ganglia and frog limb stump *in vivo*; regeneration of severed peripheral nerves and spinal cords was also noticed to proceed at increased rates in the presence of pulsed electromagnetic field [91].

However, evidence about possible mechanisms underlying the effectiveness of electrical stimulation in PNS regeneration has remained elusive so far. The most important finding in this regard is that reported by Sokoloff and collaborators [92] through stimulation of the proximal stump of a transected sciatic nerve. These authors have found that, by using the quantitative autoradiographic deoxy-D-[^{14}C] glucose method, a frequency-dependent activation of glucose utilization was detectable in the dorsal horn of the spinal cord, while cell bodies of the dorsal root ganglion showed no metabolic changes. These results suggest that axon terminals, and not the cell bodies, are the site of enhanced metabolic activity during increased functional activity of regenerating sensory pathways; analogous studies on the motor side are still lacking.

It is evident from the data summarized above that a better understanding of the neurobiological bases on which regeneration processes are founded is required in order to achieve significant fostering of nerve regeneration through agents which may play critical roles at one or another stage of regeneration itself. To investigate new agents capable of promoting neurite outgrowth, an experimental system must be designed in such a way that neurite performance is less than optimal so as to permit observation of a positive effect, and yet not so severely restricted that the putative promoting agent cannot display a positive effect. One such experimental model is the "regenerating chamber", where the proximal and distal stumps of a resected nerve are inserted at the opposite ends of tubular, preformed spaces so as to leave selected gaps between the two stumps [93]. Several materials have been utilized as constituents of the chambers, from host-made mesothelial cell walls, to silicone tubes, and a number of semipermeable polypropylene compounds [94] or bioresorbable synthetic poly-D, L-lactates rendered flexible with triethyl citrate added as a plasticizer [95]. Most of them remain unfortunately limited to the experimental use, yet in such chambers it is becoming possible: 1) to analyze the molecular components which accompany and may control the stages through which generation of a newly formed nerved structure occurs across the interstump gap; 2) to recognize the agents which may play critical roles in initiating and maintaining one or another stage of nerve regeneration; 3) to manipulate both the humoral and the cellular microenvironment in which nerve regeneration takes place.

Thus far, three main groups of agents have been recognized which could be regarded as mediators of some aspects of regenerative processes: 1) neurotrophic factors (NTFs), which are possibly concerned primarily with maintenance and repair of the damaged neurons in the early stages via retrograde axonal transport, and continue to provide a supportive role to the regrowing axons at a local level; 2) neurite-promoting factors (NPFs), which actually control axonal advance; 3) matrix-forming precursors (MFPs) supplied into the chamber from blood and nerve exudates. These latter materials, which become assembled into a coaxial matrix under the effect of regulatory molecules which are also delivered to the chamber fluid, are especially important. Indeed, it is becoming increasingly evident that fibronectin pathways underlined by a properly oriented, polymerized fibrin scaffolding is an absolute requirement for promoting and guiding Schwann cell immigration. Prefilling of a chamber with phosphate buffer saline or, better, with dialyzed plasma allows to achieve successful axonal regeneration even across gaps which were thought being too large (up to 20 mm) for a functionally and anatomically efficient reconnection of the stumps [96]. Such results entail new directions for future work on molecular mechanisms regulating early steps in nerve repair. For instance, it will be possible to alter the composition of matrix supplement in order to improve: 1) the temporal progression of intra-chamber regeneration, 2) the number of axons participating in the nerve regeneration. Additionally, it will be possible to manipulate experimentally the Schwann cell performances by interfering with fibronectin and laminin production and layout. Several studies [97-99] have shown that fibronectin is spontaneously appearing in the naturally forming matrix as early as the latter is being assembled, whereas laminin is displayed by the matrix (and by the chamber fluid) only after immigration of Schwann cells. Fibronectin contains fibrin-binding domains which allows it to anchor to a fibrin matrix (and be subsequently coupled covalently to it) and is known to promote cell migration *in vitro* and *in vivo*. This suggests that Schwann cells in the nerve stump inserts have to undergo a series of steps (probably including proliferation) before they are ready to move into the chamber matrix and to supply the latter with their neurite-promoting laminin and other neurotrophic substances reviewed in the previous sections.

According to these results, a new interpretation may be offered for the reinnervation of muscle-free basal lamina "ghosts" recently reported in several laboratories worldwide [100-103]. Even in the absence of Schwann cells, neurite outgrowth can be successfully obtained if the elongation occurs along the inside of the basal lamina tubes vacated by the Schwann cells. Since this phenomenon is absolutely dependent upon the actual existence of a basal lamina remnant and the orientation of its fibrillary glycoproteins, it can be concluded that the sequence of events underlying a functionally and anatomically repair of severed PNS fibers is as follows:

formation of a fibrin matrix → fibronectin layout → Schwann cell activation and proliferation → production of growing and myelination factors by Schwann cells → laminin and proteoglycan scaffolding → production of NTFs and NPFs → neurite outgrowth along matrix and basal lamina materials → production of muscle-derived growth factors → espression of cell-cell adhesion molecules → recognition of peripheral endplates. Obviously, most of these events occur simultaneously and are strictly interrelated.

Conclusions

The purpose of the present paper was to review recently acquired knowledge, and a large number of open questions as well, about some neurotrophic factors and mechanisms determining the success or failure of peripheral nerve regeneration after injury. It is now clear to neurobiologists that regeneration is not solely a property of the neuron, but is heavily dependent on the relationship between Schwann cell and axons which in turn is linked to the axon being in contact with a myelinating Schwann cell. Since electron microscopy has shown that growth cones of regenerating nerve fibers adhere closely to the inner surface of the Schwann cell basal lamina even after the cells within the nerve stump have been killed, it is conceivable that some components of basal lamina (laminin, heparan sulphate proteoglycan, other unidentified soluble proteins) are crucial to guide and promote the growth of axons through peripheral and intramuscular nerves [104-106]. This implies that, in order to be maximally effective, any pharmacological treatment devised for enhancing peripheral nerve regeneration and repair, must involve primarily a modulative effect on those factors, whether derived from plasma, muscle fibers, axons, activated Schwann or other non-neural cells, which play a role in neurite outgrowth, neuronal survival, and axonal guidance to the proper terminal target. A large number of substances have been claimed or demonstrated to be effective in enhancing neuromuscular regenerative processes: these are, gangliosides [107, 108], isaxonine [109], forskolin [110], nafthydrofuryl [111], pyronin G [112], α-melanocyte-stimulating hormone [113], $ACTH_{4-10}$ [114], vitamin B complex [115], cyclophosphamide [116], thyroxine and triiodothyronin [117], corticosteroids [118], spermine and other polyamines [119], testosterone [120], thyrotropin-releasing hormone [121], adenosine-3',5'-monophosphate [122], and leupeptin [123]. While a survey of these agents falls beyond the aims of the present review, and will be the topic of a further paper [124], it is suggestive noting here that for one of them, namely the gangliosides, experimental evidence is cumulating that they may act by modulating some properties of neurotrophic factors, as well as by intervening on Schwann and perineurial cells features, i.e. by modifying the microenvironment through which axons have to regrow.

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