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Efficacy of ganglioside treatment in reducing functional alterations induced by vincristine in rabbit peripheral nerves*

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Summary. Vincristine (VCR) administration to rabbits resulted in severe electrophysiologic alterations of peripheral nerves. Sciatic nerve conduction velocity, compound action potential (CAP) amplitude, and area under the CAP waveform were all reduced in a dose-dependent fashion. In addition, the pattern of conduction velocity of both motor and sensory fibers was altered and shifted toward slow conduction classes. Simultaneous treatment with gangliosides limited significantly the changes in electrophysiologic parameters induced by VCR. It is suggested that gangliosides be given in the clinical setting as protection for the peripheral nerves against the side effects of antiblastic therapy.

Introduction

Vincristine (VCR) is an important antineoplastic agent that is widely used in the chemotherapy of different types of malignancies, especially leukemias and lymphomas. At therapeutic doses, the most relevant side effect of this drug is a sensory-motor peripheral neuropathy characterized by clinical signs and symptoms such as hyporeflexia, paresthesia, muscle pain and weakness [3, 29, 31]. The neurophysiologic analysis of VCR-induced neuropathy reveals major changes in sensory nerve action potentials, generally in the form of reduced amplitude and slowed conduction velocity [3, 4, 18]. Increases in the distal motor latency and presence of denervation potentials in distal muscles have also been reported [3]. Neuropathologic examination demonstrates nerve-fiber degeneration [3], which can result in a significant fiber loss and alteration in the composition of myelinated fibers [26]. However, very often there is no direct correlation between the degree of morphologic and morphometric alterations and the severity of functional impairment [3, 16].

Based on preliminary observations that ganglioside (GA) treatment may prevent VCR toxicity [20], studies have been carried out to evaluate the effects of GA administration concomitant to VCR therapy. High-concentration (10^{-4} *M*) GA in vitro does not reduce VCR antiblastic properties, evaluated as an inhibition of N_2A neuroblastoma and C_6 glioma cell proliferation [9]. Similarly, in vivo, simultaneous GA administration does not affect VCR-induced inhibition of N_2A neuroblastoma following subcutaneous inoculation in the mouse [9].

The rationale for using GA to reduce the severity and/or incidence of VCR-induced neuropathy stems from the biochemical, physico-chemical, and pharmacological characteristics of these molecules [19, 37]. Gangliosides are glycosphingolipids that occur in nearly all cellular membranes and are particularly concentrated in nervous tissue. Exogenous GA can insert itself into plasma membranes [23], modulate enzymatic activities [5, 13, 22] and stimulate membrane functions such as active Na^+-K^+ transport [38]. When applied to neuronal cell cultures, GA enhances survival [24] and neurite growth [8, 34], probably by increasing cellular responsiveness to neuronotrophic agents such as nerve growth factor [7, 10]. Exogenous GA stimulates peripheral nerve regeneration and sprouting following mechanical injury [14, 15] and counteracts morphologic, electrophysiologic and biochemical alterations associated with different types of experimental diabetic neuropathy [1, 27, 32]. Furthermore, in induced toxic diabetes, the reduction in slow axonal transport of acetylcholinesterase, neurofilaments and tubulins can be prevented by GA treatment [11, 25].

Axonal transport disturbances, related to cytoskeletal derangement [30], are also considered to play a pivotal role in the pathogenesis of VCR-induced peripheral neuropathy [17]. Examples of cytoskeletal organelle accumulation due to VCR administration have been reported in some animal species, such as the cat [6]. More recently, reproducible peripheral-nerve pathologies induced by chronic VCR treatment in rabbits have been reported in our laboratories [28].

The present study describes the capability of exogenous GA to limit the electrophysiologic alterations that occur in the peripheral nerves of rabbits treated with VCR regimens comparable with those used in the clinical setting.

Materials and methods

Drugs. Vincristine sulfate (Lilly France S. A.; Fegersheim, France) was dissolved in saline containing 0.9% benzyl alcohol. GA, a highly purified mixture of GM_1 (21%), GD_{1a} (40%), GD_{1b} (16%), GD_3 (2%), GT_{1b} (19%) and GQ_{1b} (2%)

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(nomenclature according to Svennerholm [35]), were extracted from calf-brain cortex according to the method of Tettamanti et al. [36] and dissolved in phosphate-buffered saline (PBS).

Treatment protocol and experimental groups. The study was carried out on male New Zealand rabbits weighing 2 kg at the beginning of the experiments. Five groups of animals were used for in vitro electrophysiologic measurements, according to the following schedule:

1. The first group of rabbits ($n = 9$) was studied after a 2-week quarantine period and used as a baseline reference (time 0).

2. The second group was randomly subdivided into three subgroups and treated with (a) 0.2 mg/kg i.v. VCR once a week for a total of 5 consecutive weeks, plus 0.5 ml/kg per day i.v. PBS six times a week; (b) 0.2 mg/kg i.v. VCR once a week for a total of 5 consecutive weeks, plus 50 mg/kg per day i.v. GA six times a week; and (c) VCR solvent once a week for 5 weeks, plus 0.5 ml/kg per day i.v. PBS six times a week (control). Daily treatments with GA or PBS started 3 days before the first VCR administration and ended 2 days after the last VCR injection. At 4–8 days after the end of VCR treatment, the animals were sacrificed and the electrophysiologic properties of the sciatic nerve were assessed. The final number of animals examined comprised 6 in the solvent + PBS subgroup, 14 in the VCR + PBS subgroup and 14 in the VCR + GA subgroup.

3. The third experimental group was subdivided in a similar way, but the weekly VCR dose was raised to 0.25 mg/kg. In all, 9 rabbits in the solvent + PBS (control) subgroup, 15 in the VCR + PBS subgroup and 17 in the VCR + GA subgroup were examined at the end of the treatment period.

4. The fourth group was composed of animals receiving 0.25 mg/kg VCR + PBS or VCR solvent + PBS (control) per week. This group was further subdivided into two subgroups: after the 5-week treatment period, the first was left untreated for 2 more weeks (control, $n = 7$; VCR + PBS, $n = 12$), whereas the second was followed for 4 additional weeks (control, $n = 7$; VCR + PBS, $n = 14$) before sacrifice. Data from animals in these two subgroups were used to monitor incidental recovery of nerve functions after VCR withdrawal.

5. The fifth group of rabbits was given 0.25 mg/kg VCR + PBS ($n = 8$) or VCR + GA ($n = 9$) per week and left untreated for 4 more weeks after the last VCR injection.

Rabbits in a sixth group, treated with 0.25 mg/kg VCR per week plus either PBS ($n = 6$) or GA ($n = 6$) or with VCR solvent + PBS ($n = 6$) according to the above protocol, were used for in vivo measurement of conduction velocity of individual nerve fibers at the end of VCR treatment.

Electrophysiology. In vitro electrophysiologic measurements were carried out on sciatic nerve dissected from rabbits under deep pentobarbital anesthesia. The tibial and peroneal branches were ligated together about 5 mm proximal to the branch of the gastrocnemius nerve. Following 20 min incubation at 37° C in Krebs' solution bubbled with 95% O_2 and 5% CO_2, the nerve was placed in a thermostatic chamber on a grid made up of platinum wires spaced 2.5 mm apart. A pair of adjacent wires contacting the proximal part of the nerve were used as stimulating electrodes. At 15 mm from the stimulating electrodes, a pair of adjacent wires (proximal recording pair) recorded biphasic compound action potentials (CAP) Two additional electrodes (distal recording pair) were located 50 and 62.5 mm, respectively, from the first electrode of the proximal pair.

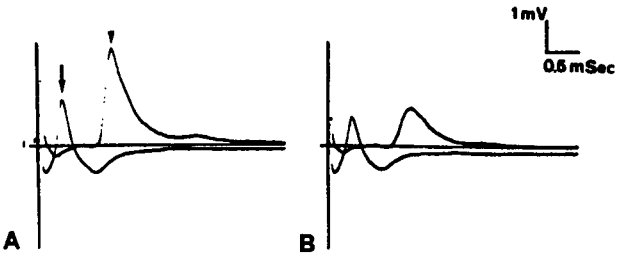

Fig. 1. In vitro recording of sciatic nerve compound action potential (CAP) in A control and B VCR-treated rabbits (0.2 mg/kg per week). Biphasic CAP (*arrow*) was recorded 15 mm distal to the site of stimulation. Monophasic CAP (*arrowhead*) was recorded 50 mm distal to the site of biphasic CAP recording. Note the decrease in the monophasic CAP amplitude and area induced by the drug

The ligature between the tibial and peroneal nerves was positioned over the second electrode of the distal pair (indifferent electrode) to enable the differential derivation of the monophasic CAP. The nerve was stimulated at 1 Hz with supramaximal square pulses lasting 25 μs, delivered by Tektronix function and pulse generators (Tektronix FG 501 and PG 505; Beaverton, Ore, USA) through a WPI 305 R stimulus insulator (WPI; New Haven, Conn, USA). In all, 30 consecutive CAPs were recorded, averaged by a two-channel differential digital oscilloscope (Nicolet 4094A; Madison, Wis, USA), stored on floppy disks, and analyzed after proper retrieval. The following CAP parameters were considered: peak amplitude, area under the compound waveform, and conduction velocity of the α-wave. The latter was calculated from the time elapsed between the peak of the biphasic CAP, recorded proximally, and the peak of the monophasic CAP, divided by the distance between the recording electrodes (50 mm; Fig. 1).

Measurement of the conduction velocity of individual sensory and motor axons was also done in vivo with the animals under deep pentobarbital anesthesia. Sural, peroneal, gluteus maximus and tibial nerve branches were all severed, with the exception of the medial gastrocnemius nerve. This nerve was covered with liquid paraffin to prevent drying, maintained at 37° C by radiant heat, and stimulated at 1 Hz with square pulses of 25-μs duration. The L7 and S1 dorsal and ventral roots were exposed by laminectomy, covered with liquid paraffin heated to 37° C, and teased into small bundles on which differential recordings were carried out using a low-noise extracellular preamplifier (EG&G model 113; Princeton, NJ, USA). Single-fiber action potentials produced after medial gastrocnemius nerve stimulation were identified, recorded on the digital oscilloscope, stored on floppy disks, and analyzed. At the end of the recording session, the nerve was removed and the distance between stimulation and recording sites was measured. Conduction velocity was then calculated as the quotient of the latency of single-fiber action potential (i.e. the time elapsed between stimulus and action-potential peak) and the conduction distance. Correction for stimulus utilization time [2] was

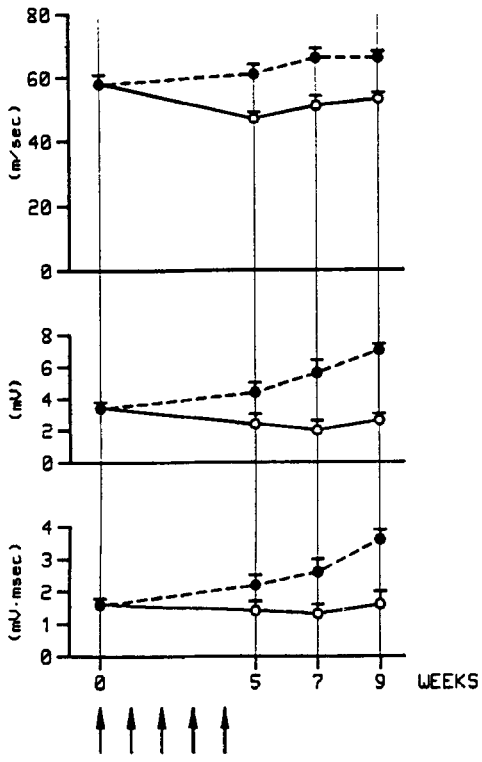

Fig. 2. Nerve conduction velocity (*upper panel*) and sciatic nerve CAP amplitude (*middle*) and area (*lower*) in control (*dashed line*) and (0.25 mg/kg per week) VCR-treated rabbits (*solid line*). *Arrows* indicate i. v. injections of either vincristine sulfate or VCR solvent alone. Time 0 values represent CAP parameters before the start of treatment

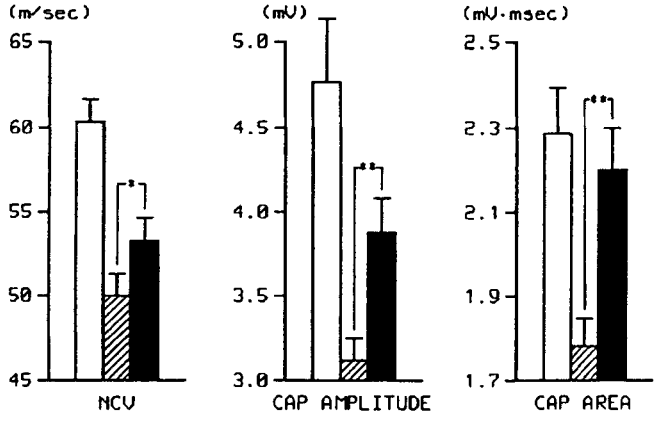

Fig. 3. CAP parameters at the end of a 5-week treatment with 0.20 mg/kg per week VCR and either PBS (*dashed bars*) or GA (50 mg/kg per day i. v., *solid bars*). Empty bars represent age-matched control rabbits

not applied. Data are presented as the means ±SEM. The significance of differences is based on the two-tailed Student's *t*-test.

Results

Effects of VCR on sciatic nerve CAP

CAP properties in control and VCR-treated (0.25 mg/kg per week) rabbits are illustrated in Fig. 2. In normal animals, although all CAP parameters increased as a function of time, the peak amplitude and the area under the waveform increased most markedly. At the end of the 5-week VCR treatment, CAP mean amplitude and α-peak conduction velocity were both reduced in comparison with the initial values (time 0). Differences were significant, with a probability $P < 0.001$. The area under the waveform was also reduced, but at a level of significance of only 7.6%. The differences were highly significant for all of the CAP parameters when VCR-treated animals were compared with age-matched controls: the area was reduced by 35%; the amplitude, by 46%; and the α-conduction velocity, by 24%.

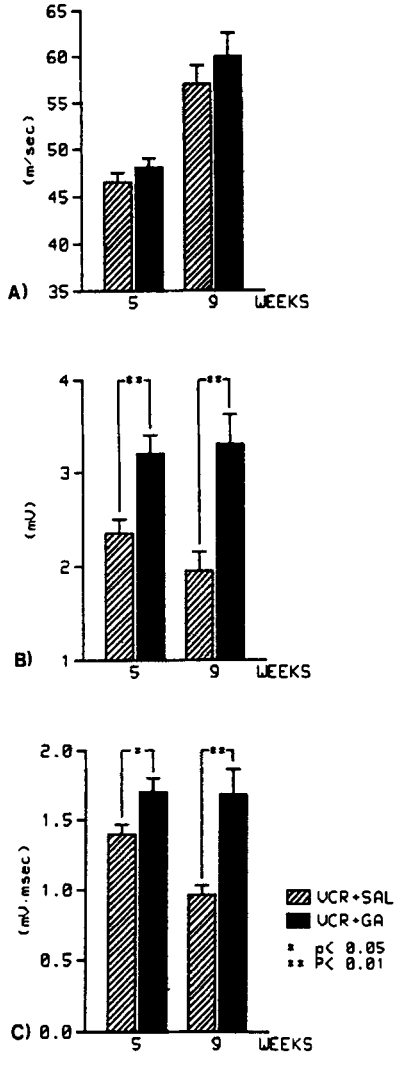

Fig. 4. A Nerve conduction velocity and CAP B amplitude and C area at the end of a 5-week treatment with 0.25 mg/kg per week VCR, compared with those measured 4 weeks later. Rabbits received in addition either GA (50 mg/kg per day i. v., *solid bars*) or PBS (*dashed bars*). All of these additional treatments were withdrawn at the end of VCR administration

At 4 weeks after VCR withdrawal, α-peak conduction velocity improved significantly ($P < 0.001$ vs the value at the end of VCR treatment). In contrast, CAP amplitude and area values were not significantly different from those recorded after the last VCR injection. As a result, the differences between VCR-treated and age-matched control rabbits were even higher 1 month after VCR withdrawal than at the end of VCR treatment.

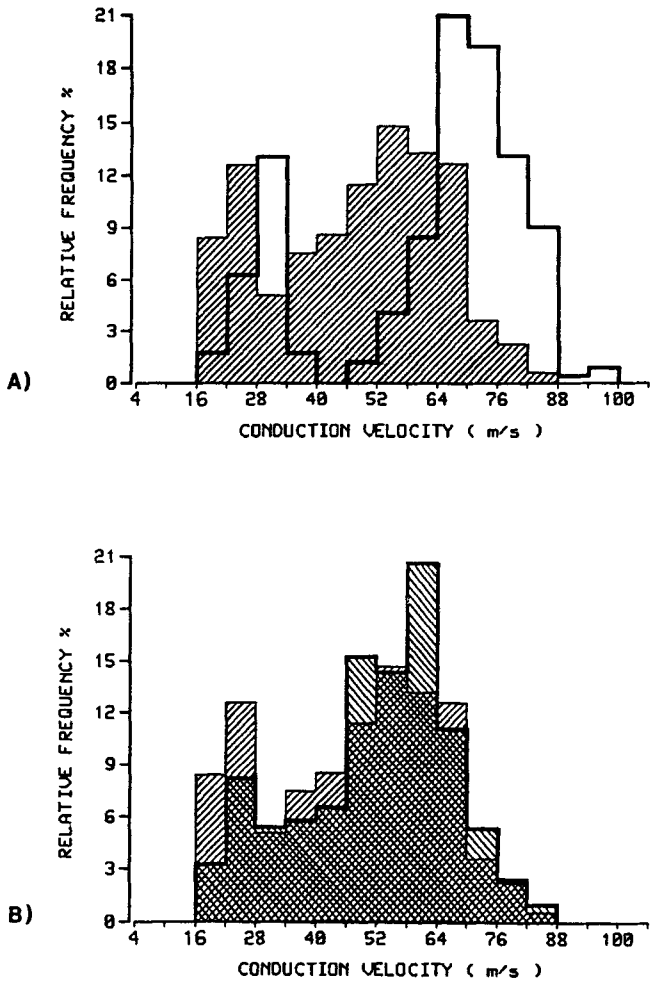

Fig. 5. Conduction velocity distribution of medial gastrocnemius nerve fibers in L7 and S1 ventral roots. A Distributions in control (*empty areas*) and in VCR-treated (0.25 mg/kg per week) rabbits (*dashed areas*) are superimposed for comparison. Note that a the bimodal distribution in normal fibers is nearly abolished after VCR treatment. B GA administration does not significantly improve any distribution class, although medium-sized fibers seem to conduct better. □ control; ▨ VCR + SAL; ▧ VCR + GA

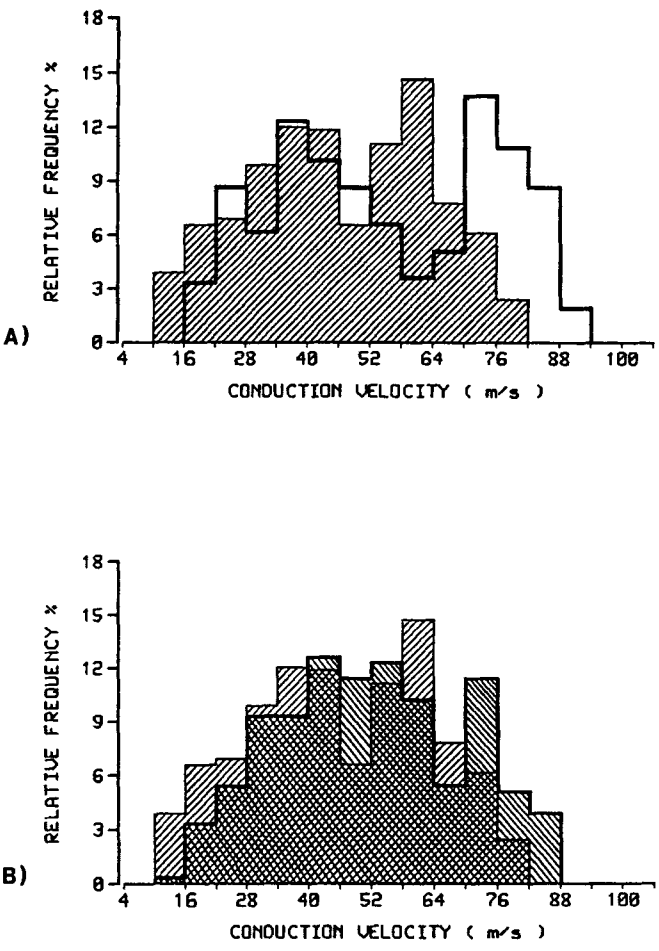

Fig. 6. A Conduction velocity distribution of medial gastrocnemius nerve fibers in L7 and S1 dorsal roots (details as in Fig. 5). B The fastest conducting fibers appear to be selectively protected by GA treatment. □ control; ▧ VCR + SAL; ▧ VCR + GA

GA prevention of CAP alterations

Rabbits treated with either 0.20 or 0.25 mg/kg VCR per week also received PBS or GA (50 mg/kg per day, i. v.). At 4–8 days after the fifth VCR injection, the animals were killed and the CAP properties were assessed in vitro. The administration of 0.20 mg/kg VCR per week + PBS reduced CAP amplitude by 35%, CAP area by 22% and α-conduction velocity by 18%, in comparison with values obtained in age-matched control animals. Rabbits receiving the same dose of VCR and additionally treated with GA generated CAPs much more similar to the control waveforms, with the CAP amplitude reduced by 19%; the CAP area, by only 4%; and α-conduction velocity, by 12%. The differences between VCR + PBS- and VCR + GA-treated animals were significant at the 1% level for CAP amplitude and area. The difference in α-conduction velocity was significant at the 5% level (Fig. 3).

GA administration also limited CAP alterations in rabbits undergoing the 0.25 mg/kg per week VCR regimen. At the end of the treatment, the decrease in CAP amplitude was reduced from 46% to 25% and that in CAP area, from 35% to 22%. Differences between VCR + PBS- and VCR + GA-treated rabbits were significant at the 1% and 5% levels, respectively. At 4 weeks after VCR withdrawal (all drug treatments ended simultaneously with the termination of VCR administration), the CAP amplitude and area were still significantly higher in GA- than in PBS-treated groups. In addition, recovery of neural conduction velocity was accelerated in GA-treated animals, although the difference in comparison with values obtained in the 0.25 mg/kg VCR + PBS-treated group was not statistically significant (Fig. 4).

Distribution of single-fiber conduction velocity

Following treatment, the pattern of single-fiber conduction velocity was examined to ascertain whether VCR neuropathy affected a selected fiber subgroup and/or whether GA efficacy was related to the protection of a specific fiber type. Conduction velocity distribution of the medial gastrocnemius nerve fibers in L7 and S1 spinal roots in control and VCR + PBS-treated rabbits are illustrated in Figs. 5 A (motor roots) and 6 A (sensory roots).

VCR (0.25 mg/kg per week) had a clear-cut effect on both sensory and motor fibers, shifting all fiber populations toward slower conduction velocities. The pathology was not selective for any fiber type.

Conduction velocity patterns in rabbits treated with 0.25 mg/kg VCR per week and receiving either PBS or GA are compared in Figs. 5 B and 6 B. Whereas only small differences were apparent in the distribution of motor-fiber conduction velocity, GA treatment was effective in protecting the population of high-speed conducting sensory fibers that were almost completely lost in VCR + PBS-treated animals. In addition, after GA treatment the overall pattern of sensory-fiber conduction velocity was closer to that of control animals. From the statistical point of view (chi-square test), the difference in conduction velocity distributions in VCR + PBS- and VCR + GA-treated rabbits was significant at the 0.1% level.

Discussion

The present study shows that the administration of VCR to rabbits results in severe alterations of CAP properties, which may be dependent on different pathogenetic mechanisms. For instance, at 0.25 mg/kg per week, the drug caused a reduction of CAP amplitude and α-peak conduction velocity and completely inhibited the processes leading to an increase in CAP area as a function of time in normal rabbits. The inhibition of functional maturation was still present 1 month after VCR withdrawal. Because it is known that the amplitude of single-fiber action potential (i.e. the elementary event contributing to CAP size and shape) is a function of fiber diameter [12], it is possible that VCR impairs a normally occurring process conducive to an increase in axonal size. This may well be related to VCR-induced disturbances in the axonal transport of cytoskeletal organelles, particularly neurofilaments, the density of which may control the axon transverse area [21].

However, the extensive reduction occurring in all CAP parameters compared with values obtained in age-matched control animals suggests that in addition to maturation impairment, the loss and/or functional inactivation of nerve fibers could be relevant to the pathogenesis of this toxic neuropathy. Ultrastructural analysis of the same nerve specimens revealed the presence of degenerating nerve fibers, both myelinated and non-myelinated, in VCR-treated rabbits [33]. Although the condition appears to be a primary axonopathy, secondary myelin alterations are often observed. Morphometric studies are now in progress in an attempt to assess the extent of structural damage (either fiber loss and/or maturational deficit) and to correlate histopathologic changes with the electrophysiologic data reported in this paper.

Consequent to VCR treatment, an analysis of patterns of single-fiber conduction velocity demonstrated a uniform decrease in conduction speed that involved all fiber subtypes in both motor and sensory roots. Reduced conduction velocity is normally attributable to myelin damage or fiber atrophy [39]. However, since the pattern is expressed as the relative frequency for each conduction velocity class, a generalized fiber loss or functional block would not be revealed by this type of analysis.

GA administration during the period of VCR treatment significantly counteracted the CAP alterations produced by the antiblastic drug. In animals given 0.20 mg/kg VCR per week, the protective effect was exerted on all CAP parameters – above all, on CAP amplitude and area, the difference in comparison with VCR + PBS-treated rabbits being significant at the 1% level. Likewise, in the group treated with 0.25 mg/kg VCR per week, GA administration markedly improved CAP area and amplitude. These effects could be related to the promotion of axon structural maturation, the maintenance of axon membrane function, or a reduction in fiber structural damage and/or degeneration.

GA treatment has proven to be effective in other peripheral nerve disorders characterized by axonal transport impairment, such as diabetic and toxic neuropathies [11, 25, 27]. Axonal transport may also be the key to GA effects on peripheral nerve electrophysiology with concomitant VCR administration. In addition, the reported effects of exogenous GA on crucial membrane-bound enzymes, such as Na^+, K^+-ATPase of the peripheral nerve [1], could favor the maintenance of physiologic ionic gradients and preserve membrane excitability.

Results concerning single-fiber conduction velocity suggest that sensory fibers were more responsive to GA treatment. GA administration succeeded in preserving a population of fast-conducting sensory fibers, which was normally lost after VCR treatment. The protection of fast-conducting, large-caliber sensory fibers could partially account for the presence of CAPs with higher amplitude and area.

Since large sensory fibers correspond to the primary afferents (Ia and Ib), the selective efficacy of GA on these fibers could have relevant clinical implications. The loss of T reflex (i.e. reflex due to the activation of muscle spindles) is, indeed, one of the earliest signs of neurological impairment during VCR chemotherapy. Therefore, current studies are focusing on the functional response of muscle spindles and Golgi tendon organs in the same experimental model to test the capability of GA administration to preserve proprioceptive organs from VCR-induced damage.

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