LETHAL RETROGRADE AXOPLASMIC TRANSPORT OF DOXORUBICIN (ADRIAMYCIN) TO MOTOR NEURONS
A TOXIC MOTOR NEURONOPATHY

by JOHN D. ENGLAND, ARTHUR K. ASBURY, EDWARD K. RHEE and AUSTIN J. SUMNER

(From the Department of Neurology, Hospital of the University of Pennsylvania, Philadelphia, USA)

SUMMARY

Doxorubicin (Adriamycin) is an autofluorescent anthracycline antibiotic that acts as a DNA intercalator. Following intraneural microinjection of 3 μg of doxorubicin into rat tibial nerve, fluorescence microscopy indicated that it is transported retrogradely to anterior horn cell bodies. Subsequently, these motor neurons underwent a progressive subacute degeneration that occurred over a period of 35–39 days. Combined electrophysiological and neuropathological methods indicated that dorsal root ganglion cells were relatively unaffected by this dose of toxin. The selective motor neuron degeneration produced by this agent raises the possibility that abnormalities of nucleic acid metabolism may be involved in the aetiology of motor neuron diseases.

INTRODUCTION

Doxorubicin is an anthracycline antineoplastic antibiotic that acts at the cell nucleus by intercalating between the base pairs of DNA, thus inhibiting DNA-directed messenger RNA synthesis (Pigram et al., 1972; Di Marco, 1975). The intraneural injection of this substance causes focal Schwann cell degeneration and consequent demyelination (England et al., 1988). With quantities of doxorubicin larger than those required to intoxicate Schwann cells selectively, this agent can undergo preferential retrograde axoplasmic transport to motor neuron nuclei, resulting in a relatively selective motor neuron degeneration. This experimental motor neuronopathy may therefore provide a model of motor neuron disease. The electrophysiological and neuropathological effects of this toxin upon motor neurons form the basis of this report.

Correspondence to: Dr Austin J. Sumner, Department of Neurology, Hospital of the University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, USA.

© Oxford University Press 1988
METHODS

Intraneural injection

Male Wistar rats (350-400 g) were used in these experiments. Doxorubicin (Adriamycin) was diluted in normal saline to a concentration of 100 μg/ml, and 30 μl of this solution (i.e., 3 μg Adriamycin) were injected into the subperineurial region of the single fascicle of the tibial nerve. The details of this procedure have been described previously (England et al., 1988). For each animal only one side was injected so that the contralateral (uninjected) side could serve as a control.

Electrophysiological studies

Compound muscle action potentials (CMAPs) were recorded from the intrinsic foot muscles after stimulation of the sciatic nerve at the sciatic notch (proximal stimulation) and the tibial nerve at the ankle (distal stimulation). The details of this procedure have also been described previously (England et al., 1988).

In 6 animals, nerve action potentials (NAPs) were recorded from the tibial nerve and its contributing dorsal and ventral roots (L4, L5 and L6) after stimulation of the tibial nerve at the ankle. For these studies the entire sciatic-tibial nerve as well as the L4, L5 and L6 dorsal and ventral roots were exposed surgically, dissected and isolated. When the surgical field was clean and free of bleeding points, the nervous tissue was covered in a paraffin bath continuously bubbled with carbogen. The temperature of the bath was maintained between 37-38°C by a thermostatically controlled heating device. A pair of platinum tipped steel electrodes (Grass E2) placed alongside the tibial nerve at the ankle were used for stimulation. Square-wave pulses of current 0.1 ms in duration were delivered at a rate of 1 Hz. DISA near-nerve recording electrodes were placed in a bipolar arrangement under the tibial nerve at the popliteal fossa and under the appropriate dorsal and ventral roots (L4, L5 and L6). These potentials were recorded on a TECA TD20 and averaged electronically using a frequency band of 20 Hz-2 kHz. These nerve conduction study arrangements are summarized in fig. 1.

Histopathological techniques

Animals for histopathological study were killed with an overdose of pentobarbital. Following transcardiac fixation with 0.1 M phosphate-buffered 4% glutaraldehyde, lower lumbar spinal cord and bilateral L4-L6 dorsal root ganglia were dissected free. Sciatic and tibial nerves on the side of injection were sometimes also obtained. The spinal cord was cut into transverse blocks from both rostral and caudal lumbar enlargement. Peripheral nerve was cut into small blocks from nerve
segments proximal, distal and at the site of injection. Dorsal root ganglia were fixed in toto. All tissue was then postfixed with 2% OsO$_4$ in a 0.1 M phosphate buffer, dehydrated in a series of graded ethanol, passed through propylene oxide and embedded in Epon 812. For light microscopy 1 µm sections were cut, mounted on slides, stained with methylene blue-azure II and counterstained with basic fuchsin.

Fluorescence microscopy

Since doxorubicin has unique autofluorescence properties (Bachur et al., 1970; Egorin et al., 1974; Arcamone, 1981; Bigotte et al., 1982a, b), it is easily localized in tissues using fluorescence microscopy. Animals used for fluorescence studies were killed by an overdose of pentobarbital. Unfixed caudal lumbar spinal cord segments (L4, L5, L6) and their dorsal root ganglia were rapidly dissected and quick frozen in isopentane-liquid nitrogen. Sections (8 µm) were cut in a cryomicrotome, mounted on slides, and cleared with a glycerin–water mixture (2:1). The specimens were examined with a Zeiss epifluorescence microscope (18F-2) with a BP 455–490 filter. Viewed in this manner doxorubicin has a characteristic orange-red fluorescence.

RESULTS

Location of doxorubicin-specific fluorescence

Doxorubicin-specific fluorescence was first detected in anterior horn cells of the caudal lumbar cord (L4–6) at 48 h following injection. At this time nuclei were just beginning to fluoresce, and the number of fluorescent nuclei was limited to a few per section. With time the fluorescence increased in intensity until it reached a maximum at 4–5 days following injection. During this period strikingly intense and distinct fluorescence was seen in the nuclei of many anterior horn cells in the L4–6 segments. Additionally, intense fluorescence was seen in the nuclei of satellite cells situated around the fluorescent anterior horn cells (fig. 2). After 5 days the fluorescence gradually faded but was still present in many anterior horn cells and surrounding satellite cells at 8–10 days following injection. At these times, however,

![Fig. 2. Fluorescent photomicrograph showing doxorubicin-specific fluorescence in an anterior horn cell nucleus and in nuclei of surrounding satellite cells at 4 days after injection of 3 µg of doxorubicin. Bar = 10 µm.](https://academic.oup.com/brain/article-abstract/111/4/915/288922/LETHAL-RETROGRADE-AXOPLASMIC-TRANSPORT-OF)
most cells showed fluorescence only at the nuclear periphery, giving a ring-like appearance in contrast to the diffuse nuclear fluorescence seen earlier. The doxorubicin-specific fluorescence was always well localized to the ventral horn ipsilateral to the injection; this fluorescence was not seen in the contralateral ventral horn.

At the same times, doxorubicin-specific fluorescence was rarely detectable, and then only weakly, in dorsal root ganglion cells from L4-6 segments.

Effects on Schwann cells

Microinjection of 3 μg of doxorubicin into rat peripheral nerve resulted first in a delayed subacutely evolving demyelinating conduction block secondary to Schwann cell degeneration. This Schwann cell degeneration has already been reported (England et al., 1988) but requires to be mentioned here because an awareness of this prolonged focal demyelination is necessary for a complete understanding of the electrophysiological studies that follow.

Effects on motor neurons

Electrophysiological studies were performed on 35 animals after intraneural injection of 3 μg of doxorubicin. Focal demyelination was followed by a subacute motor neuron-axon degeneration. Electrophysiologically this neuron-axon degeneration was recorded as a progressive decline in the distal CMAP. Distal motor axon failure occurred in some axons as early as days 13–14 postinjection, whereas others did not fail until as late as days 35–39 postinjection (fig. 3).

![Graph of serial mean distal compound muscle action potential (CMAP) amplitudes at various time points after injection of 30 μl of a 100 μg/ml concentration (i.e., total dose of 3 μg) of doxorubicin. The statistically significant decline in amplitude starts at days 13–14 (P at 0.01 using the Tukey-Kramer multiple comparison procedure) (Ott, 1984; Kramer, 1956, 1957). This decline in amplitude is not complete until day 39 (n = 35). The SD is less than 2.9 for all of these means. Correlative neuropathological observations show a concomitant anterior horn cell degeneration.](https://academic.oup.com/brain/article-abstract/111/4/915/288922/LETHAL-RETROGRADE-AXOPLASMIC-TRANSPORT-OF)
FIG. 4. Comparable photomicrographs of caudal lumbar anterior horns of the side ipsilateral to injection of 3 μg of doxorubicin (A, C) and the contralateral side (B, D). C and D are higher magnification views of the anterior horns of A and B respectively. Note the marked devastation of neuropil and decrease in the number of anterior horn cells in A and C as contrasted with the normal appearance in B and D. Both specimens 40 days postinjection. A, B, bar = 353 μm; C, D, bar = 62.5 μm.
Neuropathological studies showed that the motor axon degeneration was accompanied by progressive anterior horn cell degeneration (fig. 4). At a time when electrophysiological studies indicated complete degeneration of all motor axons previously innervating the intrinsic foot muscles (days 35–39 postinjection), spinal cords were obtained from 16 animals; 1 μm sections of L4–6 cord segments were examined in detail by light microscopy. Anterior horn cell counts were performed by counting the number of anterior horn cells with visible nucleoli in ×160 microscopic fields and comparing numbers ipsilateral and contralateral to the side of injection. These counts showed a highly significant decrease \((P < 0.001, \text{ paired Student's t test})\) in the number of anterior horn cells ipsilateral to the side of injection \((\bar{X} = 8 \pm SD 1.03)\) as compared with the contralateral control side \((\bar{X} = 17 \pm SD 1.51)\).

**Dissociation between the susceptibility of motor neurons versus dorsal root ganglion cells**

Simultaneous study of primary sensory neuron-axons showed that, at least in the minute amounts of doxorubicin that we have used, there is a dissociation in the susceptibility of primary sensory neurons as compared with motor neurons. Ten animals were studied by recording nerve action potentials (NAPs) from the tibial nerve and concomitantly recording compound muscle action potentials (CMAPs) from intrinsic foot muscles after stimulation of the tibial nerve at the ankle. During the time when motor axons of the tibial nerve underwent subacute

![Fig. 5. A representative sequence of tibial nerve action potentials (NAPs) and compound muscle action potentials (CMAPs) recorded after injection of 3 μg in 30 μl of doxorubicin into the tibial nerve. Notice that at day 63, the CMAPs are unelicitable, whereas the tibial NAP is well preserved (although, as expected, decreased in amplitude from day 1). At day 63, the tail of the tibial NAP also shows some temporal dispersion, indicating conduction through a segment of residual demyelination.](https://academic.oup.com/brain/article-abstract/111/4/915/288922/LETHAL-RETROGRADE-AXOPLASMIC-TRANSPORT-OF)
DOXORUBICIN MOTOR NEURONOPATHY

Fig. 6. A, L5 and L6 ventral root recordings ipsilateral to the injection at day 63 postinjection. Note that there is no reproducible response. B, L5 and L6 dorsal root recordings contralateral (right) and ipsilateral (left) to the injection at day 63 postinjection (same animal as in A). Note that dorsal root potentials on the left side are definitely present although decreased in amplitude and temporally dispersed in comparison to those on the right, indicating conduction through a segment of residual demyelination.

degeneration with a fall of the distal CMAP to zero over a period of 35–39 days, the NAPs recorded directly from this nerve remained well preserved (fig. 5).

Concomitant direct in vivo ventral and dorsal root studies were performed on 6 of these animals. Following distal tibial nerve stimulation, direct recordings were made from L4, L5 and L6 roots. At a time when distal CMAPs were unelicitable, ventral root potentials were also unelicitable (fig. 6A); dorsal root potentials, however, were preserved (fig. 6B). Thus at a time when there was complete degeneration of motor axons directed to intrinsic foot muscles, there was well preserved function of sensory axons representative of the same spinal segments.

Lower lumbar (L4–6) spinal cord segments with accompanying dorsal root
ganglia were examined in the 6 animals also studied electrophysiologically. Specimens were taken at days 38, 45, 55, 63, 70 and 87 postinjection. These specimens showed significant anterior horn cell loss on the side ipsilateral to injection (as described in the previous section) with no identifiable structural alteration of dorsal root ganglia from the same spinal segments and ipsilateral to the side of injection (fig. 7). Further detailed examination of dorsal root ganglia in 10 additional animals similarly injected (but whose NAPs were not recorded) confirmed these neuropathological findings. Hence the combined electrophysiological and morphological studies indicated a significant difference in the vulnerability of primary motor neurons as compared with primary sensory neurons.

**DISCUSSION**

Previous investigators have demonstrated motor neuron degeneration following both intramuscular and intraneural injection of doxorubicin. Bigotte and Olsson (1982, 1983) showed degeneration of mouse hypoglossal motor neurons following retrograde axonal transport of doxorubicin from the tongue. Yamamoto et al. (1984) demonstrated anterior horn cell and dorsal root ganglion cell degeneration after subepineurial sciatic nerve injection of 70 μg of doxorubicin.

By injecting doxorubicin in quantities much smaller than those used by these investigators and employing correlated electrophysiological and neuropathological methods, we have further refined and elaborated on these initial observations. Intraneural microinjection of 3 μg of doxorubicin is followed by retrograde axoplasmic transport of this substance to motor neuron nuclei, causing these cells to degenerate. Further, at least in the minute dosages that we have used, the motor neurons are significantly more vulnerable than sensory neurons (dorsal root ganglion cells). Since doxorubicin-specific fluorescence was rarely detected in
dorsal root ganglion cells at a time when motor neurons showed discrete and vivid fluorescence, this difference in susceptibility most likely reflects, at least in part, a difference in toxin uptake and/or retrograde axoplasmic transport between these two cell types. In contrast, intravenous injection of large doses of doxorubicin produces a selective dorsal root ganglion cell degeneration (Cho, 1977; Cho et al., 1979; Bronson et al., 1982; Eddy and Nathaniel, 1982). This is most probably a result of ready access of the toxin to these cells, which lack a blood-nerve permeability barrier. Yamamoto et al. (1984) also found dorsal root ganglion cell degeneration following injection of doxorubicin into rat sciatic nerve; however, they used 70 μg of doxorubicin and crushed the injection site. These studies demonstrate that dorsal root ganglion cells can be intoxicated if the quantity of doxorubicin that reaches their nuclei is great enough. Our demonstration of a differential intoxication of motor neurons versus dorsal root ganglion cells is not in conflict with the observations of Yamamoto et al. (1984) as we used much lower doses (3 μg) of doxorubicin administered intraneurally. Thus under appropriate circumstances, doxorubicin can produce a selective motor neuron degeneration. As the mechanism of action of doxorubicin is DNA intercalation, its cytotoxic effect on motor neurons must be a result of impaired RNA synthesis and eventually impaired protein synthesis. This effect is consistent with the delayed and progressive motor neuron degeneration that occurs after the incorporation of doxorubicin into the motor neuron nucleus.

The action of doxorubicin on motor neurons may be relevant to an understanding of the pathogenesis of human motor neuron diseases. There is already a considerable body of evidence indicating major abnormalities of neuronal nucleic acid metabolism in both human and animal motor neuron diseases. Mann and Yates (1974) first reported reduced levels of cytoplasmic RNA in anterior horn cells that occurred in parallel with decreased nuclear and nucleolar volumes. They postulated that this was related to a primary heterochromatization of nuclear DNA with resultant impaired synthesis of messenger RNA (Mann and Yates, 1974). Davidson et al. (1981, Davidson and Hartmann (1981a)) and Hartmann and Davidson (1982) likewise demonstrated a 31–42% reduction in neuronal RNA content in lumbar and cervical cord motor neurons, but not in nucleus dorsalis neurons. This reduction in RNA content was independent of disease duration and extent of cell loss. The base composition of RNA from the larger motor neurons in patients with amyotrophic lateral sclerosis (ALS) was also abnormal with a lower percentage of adenine and a lower ratio of adenine to uridylic acid (Davidson and Hartmann, 1981b). Additionally, cultured fibroblasts from ALS patients appear to show an impaired DNA repair capacity following exposure to the alkylating agent methyl methanesulfonate (Tandan and Bradley, 1985). There is also some preliminary evidence that ALS cells may be exquisitely sensitive to ionizing radiation (Kidson et al., 1983).

In the wobbler mouse, a mutant that suffers from a recessively inherited degeneration of motor neurons in the spinal cord and brainstem, Murakami et al.
(1980, 1981) have found several abnormalities in spinal motor neurons that suggest abnormal RNA metabolism. Specifically, they found decreased RNA content, decreased synthesis of nuclear and nucleolar RNA, decreased protein synthesis, and increased protein turnover.

Because of these and other observations, Bradley and Krasin (1982a, b) have advanced the hypothesis that the primary abnormality in ALS is the accumulation of abnormal DNA, which results from abnormal or deficient DNA repair mechanisms. Transcription of messenger RNA and thus protein synthesis would be disrupted, eventually causing motor neuron degeneration. Our study provides some support for this hypothesis, for it shows that an agent that directly interferes with nucleic acid metabolism can cause a selective motor neuron degeneration. Such models of toxic derangement of nucleic acid metabolism may indeed provide useful insights into the pathogenesis of the human motor neuron diseases.

Recently there has been a resurgence of interest in the possibility of toxic causes of degenerative disease, including ALS. ALS previously occurred in high incidence (annual incidence of 50/100,000 in the 1950s) among the Chamorro people of Guam; however, over the past 30 years, the incidence of this disease has been steadily declining and present rates (annual incidence of < 5/100,000) are only slightly greater than those in the continental United States. This rapid near disappearance of this high incidence focus strongly suggests that environmental factors were aetiological (Garruto et al., 1985). After noting that cycad, Cycas circinalis, was an important source of food for the Chamorros, Kurland (1972) had previously suggested that a toxin from this plant could cause ALS. Recently, Spencer et al. (1986) have shown that L-BMAA (beta-N-methylamino-L-alanine) from the cycad seed can cause motor neuron degeneration. Daily feeding of macaques with BMAA causes Betz cell and anterior horn cell degeneration. Thus neurotoxic agents deserve serious consideration as potential causative agents of ALS on Guam and possibly also elsewhere.

Another intriguing aspect of the doxorubicin model relates to the cell to cell transfer of this toxin. Our fluorescent studies suggest that doxorubicin moves from anterior horn cells to surrounding satellite cells. The marked devastation of ventral horns in our chronic spinal cord specimens also implies that not only anterior horn cells but also surrounding cellular elements have degenerated. Based on these observations, the transfer of doxorubicin to central motor neurons making monosynaptic contact with anterior horn cells is a distinct possibility. If such interneuronal transfer of toxicity occurs, it would further validate the usefulness of doxorubicin in producing a model of motor neuron disease and could provide new insight into the action of other nervous system toxins.

ACKNOWLEDGEMENTS

This work was supported by a Muscular Dystrophy Association research grant to the Peripheral Nerve Physiology Laboratory of the Henry M. Watts Jr Neuromuscular Disease Research Center.
J.D.E. was supported by a Muscular Dystrophy Association Postdoctoral Research fellowship. The authors also wish to thank Cathy England for help in the preparation of the manuscript.

REFERENCES


(Received May 18, 1987. Revised October 14, 1987. Accepted November 6, 1987)