Cholinergic Function in Lumbar Aluminum Myelopathy

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Abstract. To determine whether perikaryal neurofilamentous accumulation in cholinergic neurons is associated with a deficit in cholinergic function, we developed a new model of aluminum-induced neurofibrillary degeneration, referred to as focal lumbar aluminum myelopathy. The model is produced by direct intramedullary microinjection of AlCl3, which results in a characteristic neurological syndrome. Four weeks after injections, affected rabbits show extensive neurofilamentous lesions of both large and small neurons in the lumbar spinal cord, including a majority of anterior horn cells. These animals are capable of long-term survival. Posterior tibial nerve morphometry in these rabbits revealed no significant loss of myelinated fibers. Choline acetyltransferase (ChAT) activity in the sciatic nerve was decreased 39%, from 45.70 ± 2.36 nmol ACh/hour/3-mm segment in acid-injected controls to 17.72 ± 1.94 in aluminum-intoxicated rabbits. The rate of accumulation of ChAT activity proximal to a sciatic nerve ligature was significantly greater in the aluminum-treated rabbits, although the total amount of ChAT activity accumulating in a 24-hour period did not differ from controls. We conclude that aluminum-induced accumulation of neurofilaments in cholinergic perikarya is associated with a sharp decrease of ChAT activity in the axons of those cells and possibly with a compensatory increase in the rate of delivery of the enzyme.

Key Words: Aluminum, Alzheimer's disease, Axonal transport, Choline acetyltransferase, Cholinergic function, Neurofibrillary degeneration, Neurofilaments.

INTRODUCTION

The possibility that there may be a specific relationship between diseases with fibrillar degeneration of cerebral neurons and a deficit of cholinergic synaptic transmission has been raised in several studies by different laboratories. This association was first postulated when three groups of investigators (1-3) independently found that choline acetyltransferase activity was markedly decreased in senile dementia of the Alzheimer type (Alzheimer's disease), the morphological hallmark of which is neurofibrillary degeneration of neuronal cell bodies and processes. The hypothesis was strengthened by the finding that the nucleus basalis of Meynert and the medial septal nucleus showed marked neuronal loss in Alzheimer's disease (4-6), with many surviving cells containing neurofibrillary tangles. The neurons of the nucleus basalis of Meynert appear to be the cells of origin of virtually all cholinergic fibers innervating cerebral neocortex (7, 8), and the medial septal nucleus the principal
source of cholinergic input to the hippocampus (9). Furthermore, such human de­
mentias as dementia pugilistica and the dementia of Parkinson's disease have re­
cently been reported to show loss of cholinergic neurons as well as neurofibrillary
degeneration of surviving perikarya in the nucleus basalis (10). In contrast, patients
with the dementia of Huntington's disease, which is not associated with neurofi­
brillary degeneration, are spared the loss of cholinergic neurons of the nucleus
basalis (11).

Aluminum-induced encephalomyelopathy in rabbits and cats has been employed
for almost two decades as an experimental model of human neurofibrillary diseases.
The filamentous accumulations found in the neuronal perikarya of aluminum-treated
animals have been shown on ultrastructural (12), biochemical (13), and immuno­
cytochemical (13, 14) grounds to consist of apparently normal neuronal intermediate
filaments. In contrast, the paired helical filaments found in neurofibrillary tangles
and the neurites of senile plaques in Alzheimer's disease are ultrastructurally distinct
from normal neurofilaments in that they are composed of helically wound pairs of
10-nm filaments (15-17). Alzheimer filaments also differ from normal and aluminum­
induced neurofilaments biochemically, in that they are resistant to solubilization in
sodium dodecyl sulfate (SDS), urea, reducing agents, and various other harsh de­
naturants (18), and to degradation by proteolytic enzymes in vivo (Ihara and Selkoe,
unpublished observations).

In spite of these distinctions, aluminum encephalomyelopathy remains a useful
model of perikaryal neurofilamentous accumulation. Identification of an associated
cholinergic deficit in this model might strengthen its usefulness and its relevance to
human neurofibrillary disease. Previous studies have reported conflicting results
about a decrease of the cholinergic synthetic enzyme, choline acetyltransferase
(ChAT), in aluminum-treated animals (19, 20). We have undertaken the present study
to determine whether a loss of ChAT activity occurs in the axons of cholinergic
neurons affected by perikaryal neurofilamentous tangles and, if so, whether it might
result from a defect in axonal transport of the enzyme. To answer these questions,
we have developed a new chronic model of aluminum myelopathy in the rabbit,
employing selective lumbar intramedullary microinjections of aluminum chloride.

MATERIALS AND METHODS

Experimental Model

New Zealand white rabbits weighing approximately 4 kg were maintained in accordance
with the guidelines of the Committee on Animals of the Harvard Medical School and those
prepared by the Committee on Care and Use of Laboratory Animals of the Institute of
Laboratory Animal Resources, National Research Council (DHEW publication No. [NIH]
78-23, revised 1978).

Rabbits were deeply anesthetized with intravenous sodium pentobarbital administered
through a marginal vein of the ear. A small skin incision was made over the lower lumbar
vertebrae. Aliquots of 2.25 μmole of sterilized aluminum chloride in 3 μl of water were
injected transdurally into each of three intervertebral spaces between spinal segments L4 and
S1. This solution had a pH of 2.35. Control rabbits were injected in an identical fashion with
3 μl of 4.5 × 10⁻¹ N HCl, pH 2.35. All injections were performed over approximately five
minutes (min) with a hand-held 10 μl Hamilton syringe. Penetration of the spinal cord was
often associated with a hindlimb twitch. About 10% of the aluminum-injected rabbits had
hindlimb paralysis immediately postinjection and were thus excluded from further study. Two
weeks (wk) following the first set of injections, the rabbits were reinjected in an identical

manner and then maintained for two to six wk. Rabbits were killed once the full neurological syndrome of focal lumbar aluminum myelopathy developed, which usually occurred about four wk after the initial injection (two wk after the second injection).

Morphology

Spinal cords from all experimental and control animals were immersion-fixed in buffered formalin; multiple cross sections were made and stained by the Bodian procedure. Six sciatic nerves each from the aluminum-treated and control groups were studied from rabbits perfused with three liters of room-temperature 2.5% glutaraldehyde/2.0% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. In these animals, sections of the posterior tibial nerve at the level of the popliteal fossa were dissected and then post-fixed in 2% osmium tetroxide, dehydrated, and embedded in Spurr's low-viscosity epoxy resin. Semi-thin sections were made on a Reichert Ultracut and stained with toluidine blue. Thin sections cut with a diamond knife were stained with uranyl acetate and lead citrate and examined on a Philips 200 electron microscope. Myelinated fibers were counted in prints of 1,000 x magnification, using standardized random fields. Total nerve cross-sectional areas were calculated by weighing the photographs of the total endoneurial area. These values were used to calculate the total number of myelinated fibers in the nerve. The number of degenerating axonal profiles was also counted.

Choline Acetyltransferase Assay and Ligature Studies

ChAT was assayed according to the modified radiochemical method of Fonnum (21). For determination of absolute amount of ChAT activity in unligated nerves, 3-mm segments of the sciatic nerve from the sciatic notch to the popliteal fossa were assayed in aluminum-treated and acid-injected rabbits.

Two wk following the second series of injections, animals with definite clinical evidence of aluminum-induced lumbar myelopathy (see below) were utilized for sciatic nerve ligation studies. Under intravenous pentobarbital anesthesia, the sciatic nerve was exposed about 2 cm distal to the sciatic notch and a 4-0 nylon suture was tied around the nerve. The ligature was left in place for 24, 36, or 48 hours (h). When the rabbits were killed, a 3-mm segment of nerve was taken immediately proximal to the ligature, to determine the accumulation of ChAT activity. For determining the baseline ChAT activity of the ligated nerve, another 3-mm segment was taken at a point greater than 9 mm proximal to the ligature. These values were used to calculate the rate of accumulation of ChAT activity according to the method of Rasool and Bradley (22); the result is referred to as the apparent rate of axonal transport because a shift from the non-mobile to the mobile pool of axonal transport could affect the value. The rate formula (22) is as follows:

\[
\text{Rate of accumulation} = \frac{\text{activity in ligated segment} - \text{activity in baseline segment}}{\text{activity in baseline segment}} \times \frac{\text{length of segment (mm)}}{\text{hours after ligation}} \times 24 \text{ h}.
\]

The amount of accumulated enzyme activity was calculated as follows:

\[
\text{Amount of enzyme activity accumulated} = \frac{\text{activity in ligated segment} - \text{activity in baseline segment}}{\text{hours after ligation}} \times 24.
\]

RESULTS

Neurological Features of Aluminum-Induced Myelopathy

Two to three wk after the first injection, aluminum-treated animals developed a distinct clinical syndrome. At this time, a second series of injections was given which, over the ensuing two to three wk, accentuated the deficits resulting from the first injection.

The neurological features of focal lumbar aluminum myelopathy were as follows. At the beginning of the syndrome, repeatedly tapping the coat over the low back induced a superficial muscle contraction or startle. This also occurred in control rabbits, but disappeared after one or two taps. In the aluminum-treated rabbits, the response did not disappear with repetitive taps, but actually spread to the deeper limb musculature, producing a stronger motor response. We referred to this response as stimulus-induced segmental myoclonus. The response could be elicited only below the high lumbar level. In rabbits with very prominent stimulus-induced myoclonus, repeated tapping could drive the hindlimbs into total, sustained extension. This abnormal posture was maintained for one to two min beyond the cessation of the stimulus. We speculate that this response may represent a local spinal disinhibition and may correspond to a Renshaw cell dysfunction.

Jittery and ratchet-like movements of the hindlimbs were also evident within two to four wk of injection. Such movements were not elicited by passive flexion or extension of the hindlimb, but purposeful movements lost the smooth continuity of normal motion. Some difficulty in initiating movement also seemed to be present. Weakness of the hindlimbs, however, was not prominent, and the rabbits remained capable of effective hopping. Many rabbits also developed some degree of fecal or urinary incontinence, or both, after the appearance of the motor syndrome. No animals died from this syndrome.

Sensory impairment was clearly present in all the affected rabbits. There was little if any withdrawal to deep painful stimuli administered to the hindlimbs. The hindlimbs could be positioned into sustained abnormal postures, which were also assumed spontaneously at times. We speculate that this finding may represent a joint-position sense abnormality. The rabbits also lost their tendency to groom their coats below the high lumbar level.

Neuropathology of Aluminum-Induced Lumbar Myelopathy

Multiple coronal sections of lumbar and sacral spinal cord were prepared from all rabbits and stained by the Bodian method. The neurological syndrome described above always correlated with the presence of extensive neuronal perikaryal neurofibrillary lesions throughout the anterior gray matter (Fig. 1). No consistent pattern of involvement of motor nuclei within the anterior horn was apparent. Some rabbits, however, showed more extensive tangle formation in the medial motor nucleus than in the lateral motor nucleus. Selkoe et al (13) previously reported that the lateral motor nucleus was usually unaffected when aluminum was administered intracisternally. It was this sparing that led us to abandon the intracisternal route for these sciatic nerve transport studies and develop the present model. Not only were almost all medium- and small-sized neurons in the anterior horns affected, but about 80% of the large anterior horn cells of the motor nuclei showed neurofibrillary tangles. Many cells had a homogeneous hyalinized appearance and were so densely packed.
with neurofilaments that Nissl substance was not apparent. When examined electron microscopically, spinal neurons including the anterior horn cells were densely packed with 10-nm straight filaments. As recently described by Troncoso et al (26), proximal axonal swellings were also detected in this model on plastic-embedded, 1-μm-thick toluidine blue-stained sections from perfused rabbits. Swellings occasionally extended along a major portion of the course of the axon within the ventral horn. Some proximal enlargements appeared to be in direct contiguity with the perikaryon and involved the axon hillock and the proximal segment of the neuron.

No obvious neuronal loss was present on light microscopic examination of multiple cross sections from aluminum-injected rabbits compared to acid-injected controls. From animals perfused with fixative two wk after the second aluminum injections, toluidine blue-stained sections of the posterior tibial nerve at the level of the popliteal fossa were studied quantitatively (Fig. 2). Total axonal counts and axonal densities did not differ significantly between aluminum and control animals. The total number of myelinated axons per posterior tibial nerve averaged 9,551 ± 1,433 in the aluminum-treated rabbits and 8,987 ± 718 in the controls (.5 > p > .4). The posterior tibial nerve axonal densities were 10,333 ± 1,567 axons/mm² in the aluminum-treated rabbits and 11,080 ± 998 axons/mm² in the controls (.4 > p > .3). A small but significant increase was found in the total cross-sectional area of the posterior tibial nerves of the aluminum-treated animals. Mean axonal area in aluminum-treated rabbits was 0.9302 ± .0323 mm² (0.02 > p > .01). Counts of degenerating axonal profiles indicated a small, but statistically significant, increase in axonal degeneration in the aluminum-treated animals (Table I); however, even in the most severely affected rabbits, shown in Figure 3, the number of degenerating axons reached only 2% of the total number.

Fig. 1. Aluminum-induced neurofibrillary tangles. Bodian stain of rabbit lumbar spinal cord after a microinjection of AlCl₃. The large anterior horn cells, nearly all of which contain abundant neurofibrillary tangles, make up the lateral motor nucleus. × 500.
of sciatic nerve axons. Electron microscopic sections of the posterior tibial nerve at the level of the popliteal fossa in aluminum-treated rabbits occasionally showed early axonal degeneration. As seen in Figure 4, in an animal which received only a single AlCl₃ injection and was perfused four wk later, there is a largely intact myelin sheath with preserved periodicity and focal collapse of the underlying axoplasm. These changes four wk after injection indicate an ongoing pathologic process which was not seen in control animals. While a small amount of axonal degeneration must be included as a feature of selective lumbar myelopathy, it does not result in a significant loss of sciatic nerve axons.

Sciatic Nerve ChAT Activity

Three-mm segments were taken along nearly the entire course of the sciatic nerve. ChAT activities among all segments in one nerve did not vary significantly. The mean ChAT activity per 3-mm segment was determined for the entire nerve, and these values are listed in Table 2 for all control and aluminum-treated animals. There is no overlap between these sets of values (p < .001). The mean value for the entire

<table>
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<th>Axonal Degeneration in Al⁺⁺-Treated Rabbits</th>
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<tr>
<td>Control</td>
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<td>Al⁺⁺</td>
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aluminum-treated group (17.72 ± 1.94 nmol ACh/h/mm segment) is 39% of control values (45.70 ± 2.36) (Table 2).

**Rate of ChAT Activity Accumulation Proximal to Sciatic Nerve Ligature**

ChAT activity was determined in a 3-mm segment just proximal to a sciatic nerve ligature and in a baseline segment greater than 9 mm proximal to the ligature. The latter segment did not differ significantly from values even more proximal or far distal to the ligature. Most rabbits were killed 24 h after the ligature was placed. Those rabbits with ligatures in place for 36 or 48 h continued to accumulate ChAT activity in proportion to the duration of the ligation. In one animal examined 72 h after ligation, there was no further accumulation of ChAT activity compared to 48 h.

The mean rate of accumulation (or apparent transport rate) was increased more than 200% in the aluminum-treated rabbits. The mean rate was 1.9 ± 0.36 mm/24 h in acid-injected controls and 4.1 ± 0.78 mm/24 h in the aluminum-treated group, a difference which was significant (p < 0.02). The total amount of enzyme accumulated in 24 h, however, did not differ significantly in the two groups. The mean values for the aluminum and control groups were 23.9 ± 12.4 µmol ACh/mm segment and 29.3 ± 16 µmol ACh/mm segment.

**DISCUSSION**

In a previous study, Selkoe et al (13) reported that administration of AlCl₃ to rabbits by the intracisternal route tended to spare a majority of the more lateral
Fig. 4. Early axonal degeneration associated with focal lumbar aluminum myelopathy. Electron micrograph of posterior tibial nerve axon from a rabbit given a single injection of AlCl₃ four wk previously. There is early degeneration and a focal area of collapse of the axoplasm (arrow). The periodicity of the myelin sheath is retained (inset), despite the presence of myelin whorls (arrow-heads), representing early phases of degeneration. Bar = 1 μm (main figure) and 150 nm (inset).

anterior horn cells, particularly those in the lateral motor nucleus. Because our present interest is the study of ChAT activity and transport in the sciatic nerve axons of these motor neurons, we sought to achieve more extensive involvement of anterior horn cells with neurofibrillary lesions. We, therefore, produced a selective

<table>
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<th>Control</th>
<th>Sciatic Nerve ChAT Activity*</th>
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<tr>
<td>49.58</td>
<td>19.28</td>
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<tr>
<td>48.20</td>
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<td>35.37</td>
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<td>44.10</td>
<td>11.46</td>
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<td>44.47</td>
<td>16.41</td>
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<tr>
<td>Mean: 45.70 ± 2.36</td>
<td>17.72 ± 1.94*</td>
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* In nmol ACh per h per 3-mm segment.
† Statistically different from control (p < 0.001).

lumbar aluminum myelopathy which results in the presence of tangles in more than 80% of all anterior horn cells, including the lateral motor nucleus (Fig. 1). The microinjection of aluminum chloride directly into the lumbar spinal cord produces a focal neurofibrillary myelopathy. The neurologic abnormalities in the lumbosacral region described above correlate well with the extent of neurofibrillary tangles seen histologically and therefore permit careful clinical monitoring of the development of the lesions. Because our rabbits did not develop seizures or other generalized neurologic dysfunction, the model permits long-term survival of experimental animals, an important advantage for axonal transport studies. The experiments described here were performed on animals four to eight wk after initial aluminum injections. However, other animals in our colony have survived for as long as five months, and filamentous lesions were still present. A model in which aluminum hydroxide was introduced subdurally in the lumbar spinal canal of cats has been employed by Romero and colleagues (23) for morphological studies. A chronic model of aluminum neurofibrillary disease in which a slurry of aluminum powder is injected into the subarachnoid space has been described by Wisniewski and colleagues (24).

Despite chronic survival with permanent neurological deficits, our animals did not display any obvious loss of large anterior horn cells by microscopic examination. The absence of significant anterior horn cell loss was substantiated by axonal counts. This more rigorous quantitative determination indicated that there was no significant difference in either total myelinated fiber counts or axon densities between aluminum-treated animals and controls. When degenerating axonal profiles were specifically counted (Table 1), there was a significant increase in their number in the aluminum groups compared to controls. However, because the number of such degenerating profiles was always very small relative to the total number of axons, it did not significantly alter the total axonal counts. We conclude that a small but statistically significant amount of axonal degeneration is associated with focal lumbar aluminum myelopathy. We also found a significant increase in the total cross-sectional area of the posterior tibial nerve in aluminum-treated animals. This change most likely represents endoneurial edema secondary to the axonal degeneration, although a histogram of fiber diameters would be necessary to make certain that there has not been a shift of the fiber population from smaller- to larger-diameter axons.

Ultrastructural studies on sciatic nerves in this model suggest that neither the degenerating axonal profiles nor those fibers with thinned myelin, i.e., regenerating fibers, appear to have an excess of neurofilaments. Some of the degenerating fibers seen in the aluminum-treated rabbits appeared to be in the early phases of axonal degeneration, in that the myelin around the axon retained its periodicity while the underlying axoplasm was degenerating (Fig. 4). This finding, along with the increased numbers of degenerating fibers in the aluminum group, suggests that the axonopathy associated with focal aluminum lumbar myelopathy is an ongoing process, and not due to the trauma of the injection itself.

In the support of an association between aluminum-induced neurofibrillary accumulation and cholinergic abnormality, a statistically highly significant reduction in total choline acetyltransfense activity was found in the sciatic nerves of aluminum-treated rabbits. The normal number of myelinated axons shows that this reduction could not be accounted for by a loss of ChAT-containing axons. It is also known that aluminum ions have no appreciable effect on in vitro ChAT activity.
determinations (19, 20). Thus, the marked loss of sciatic nerve ChAT activity associated with aluminum-induced neurofilamentous tangle formation in cholinergic perikarya may be due to decreased synthesis, decreased loading of enzyme onto the transport system, decreased axonal transport, increased degradation or decreased distal turn-around of the ChAT enzyme in aluminum myelopathy. A reduction in static axonal enzyme levels usually parallels the loss of axons; the reduction of axonal ChAT in the aluminum-treated rabbits is dissociated from loss of myelinated axons.

We found an increased rate of accumulation of ChAT activity proximal to a sciatic nerve ligature in animals with aluminum-induced neurofibrillary bundles. The axonal transport studies of ChAT described here used the single ligature technique, which only permits an estimate of the transport rate. In addition to the well-recognized problems of studying axonal transport of ligature techniques, the determination of the absolute transport rate requires calculation of the proportion of the enzyme which is moving (percent mobile enzyme) (22) by the double ligature placement. In the normal rat, this proportion is about 8–12% (25, and Rasool and Bradley, unpublished observations). The increased rate of ChAT accumulation in our aluminum-treated single ligature animals may indicate either an actual increase in the rate of axonal transport or an increase in the mobile pool of ChAT. In the filamentous neuropathy produced by the neurotoxin acrylamide, it has been shown that the relative distribution of axonal acetylcholinesterase can shift from the non-mobile to the mobile pool (22). The latter study showed that the mobile fraction of AChE increased from 8.1% to 11.8%. A possible shift of ChAT activity from non-mobile to mobile pools may occur in the aluminum-treated rabbits in response to the marked reduction in absolute amounts of the enzyme in the axon. Whatever the mechanism, a compensatory response is suggested because the apparent rate of transport increases in the aluminum-treated animals and the total amount of ChAT activity transported in 24 h does not significantly differ from controls. That is, despite the reduced absolute ChAT activity at any given point in the axon, the neuron apparently transports a normal total amount of ChAT activity distally. Aluminum-induced neurofibrillary swellings have been reported to begin as early as one to two days after injection (26). However, delivery of ChAT was still maintained at four wk following the aluminum intoxication in our study. Thus, cholinergic neurons containing aluminum-induced neurofibrillary tangles appear able to maintain distal delivery of the synthetic enzyme, despite the fact that their perikarya contain an abnormal accumulation of neurofilaments.

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REFERENCES


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