MORPHOMETRIC ANALYSIS OF NEURODEGENERATIVE CHANGES INDUCED BY LOW CALCIUM-MAGNESIUM AND EXCESSIVE ALUMINUM INTAKE

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ABSTRACT

To evaluate a possible role of minerals in the central nervous system degenerative process, experimental primates were fed on diet with mineral contents similar to those found in soil and water in the Western Pacific foci of amyotrophic lateral sclerosis (ALS). Monkeys were grouped according to diet of 1) Ca,Mg-deficient, 2) Ca,Mg-deficient and Al surplus, 3) Al surplus only, and 4) controls. Morphometric analyses indicate that cellular and nuclear surface areas of lumbar anterior horn cells are significantly decreased with Ca,Mg-deficient diet, and this was particularly notable in the Ca,Mg-deficient with Al surplus group. In contrast, cellular and nuclear areas are more diverse in size, being distributed in small, intermediate and large sized regions. The size distribution in the Al surplus group is similar to that in the control group. The number of axonal swellings in lumbar and cervical anterior horn tissues in the experimental groups was increased with the occasional appearance of chromatolytic neurons. These results strongly suggest that the degenerative process of motor neurons in monkeys can be accelerated by prolonged deficient intake of Ca and Mg with excess exposure to Al.

The etiology of amyotrophic lateral sclerosis (ALS) remains unknown and none of the animal models provides an exact animal copy of a specific human motor neuron disease (11). Recent epidemiological studies in the Western Pacific foci and other areas have increasingly shown the importance of environmental factors in its causation (2, 8, 9, 12). We previously reported the development of degenerative changes in the spinal motor neuron in primates fed for 6 months to a year under similar mineral conditions as seen in the ALS foci, where main findings were chromatolytic spinal neurons with eccentric nuclei, increased number of argyrophilic bodies, and accumulation of maloriented 10-nm neurofilaments in swollen axons (13). These findings were similar to the initial changes in spinal motor neu-

rons in the ALS process (5, 6), although neuronal cell loss has not yet been observed.

The present morphometric study was conducted to examine the number of argyrophilic bodies, and cellular and nuclear surface areas of spinal motor neurons in experimental primates.

MATERIALS AND METHODS

As previously reported (13), Japanese monkeys (*Macaca fuscata*) of about 10 years of age were divided into four groups: two Ca,Mg-deficient (Ex-1a, b), two Ca,Mg-deficient and Al surplus (Ex-2a, b), Al excess only (Ex-3) and two control monkeys (C-1a, b) fed for a year under the diet condition summarized in Table 1.

Table 1 *Diets in Groups of Monkeys*

Experimental groups	Minerals in 100 g of food		Water
Ca, Mg-deficient (Ex-1a, b)	Ca	0.05 g	Distilled water (D.W.)
	Mg	0.07 g	
	Al	0.004 g	
Ca, Mg-deficient and Al surplus (Ex-2a, b)	Ca	0.05 g	Aluminum lactate (3.26 g/1,000 ml D.W.)
	Mg	0.07 g	
	Al	0.004 g	
Al surplus only (Ex-3)	Ca	0.82 g	Aluminum lactate (3.26 g/1,000 ml D.W.)
	Mg	0.2 g	
	Al	0.004 g	
Control (C-1a, b)	Ca	0.82 g	Tap water
	Mg	0.07 g	
	Al	0.004 g	

Specimens for light microscopic examination were taken from cervical (C₄) and lumbar (L₃) spinal cord fixed in 10% formalin solution. Four serial paraffin sections (10 μ m thick) were mounted on a slide glass and stained with the hematoxylin-eosin, Klüver-Barrera or modified Bielschowsky stainings.

Quantification was done at a magnification of 400. The total number of argyrophilic bodies was counted in both anterior horns of a section stained with the modified Bielschowsky method. Longitudinal diameter of each body was measured using an ocular

micrometer inserted in a light microscope. In a transverse section of the lumbar spinal cord (L₃) stained with the Klüver-Barrera staining, the cellular and nuclear surface areas of all neurons in both anterior horns were measured from the photographs projected on a computed digitizer system.

RESULTS

Morphometry of Argyrophilic Bodies

The longitudinal diameters of argyrophilic bodies are shown in Tables 2 and 3. In the

Table 2 *Longitudinal Diameters of Argyrophilic Bodies in the Cervical Spinal Cord of Experimental Monkeys*

No.	Diameter (μ m)	Frequency					
		Ex-1a	Ex-1b	Ex-2a	Ex-2b	Ex-3	C-1a
1	0.00- 2.00	0	0	0	0	0	0
2	2.00- 4.00	0	0	0	0	0	0
3	4.00- 6.00	13	2	0	10	5	0
4	6.00- 8.00	31	4	7	9	7	0
5	8.00-10.00	13	7	7	8	16	5
6	10.00-12.00	7	5	6	5	14	3
7	12.00-14.00	4	3	5	7	9	2
8	14.00-16.00	6	5	7	2	9	0
9	16.00-18.00	2	1	5	0	0	1
10	18.00-20.00	0	0	1	0	1	0
11	20.00-22.00	2	0	0	0	1	0
12	22.00-24.00	0	0	0	0	0	0
13	24.00-26.00	0	0	0	0	0	0
14	26.00-28.00	0	0	0	0	0	0
15	28.00-30.00	0	0	0	0	0	0
Total number		78	27	38	41	62	5
Mean diameter		8.42	10.26	9.34	8.37	10.32	12.00
SD		3.67	3.30	3.69	3.06	2.23	2.53
Min.		4.00	5.00	4.00	4.00	4.00	7.00
Max.		20.00	17.00	16.00	14.00	20.00	16.00

Table 3 Longitudinal Diameters of Argyrophilic Bodies in the Lumbar Spinal Cord of Experimental Monkeys

No.	Diameter (μm)	Frequency					
		Ex-1a	Ex-1b	Ex-2a	Ex-2b	Ex-3	C-1a
1	0.00- 2.00	0	0	0	0	0	0
2	2.00- 4.00	0	0	0	0	0	0
3	4.00- 6.00	5	6	0	9	6	0
4	6.00- 8.00	22	19	5	9	19	4
5	8.00-10.00	15	8	5	7	10	1
6	10.00-12.00	30	14	2	6	4	0
7	12.00-14.00	26	4	1	9	9	1
8	14.00-16.00	9	16	1	3	6	1
9	16.00-18.00	6	3	0	3	5	0
10	18.00-20.00	0	2	0	1	4	0
11	20.00-22.00	0	3	0	3	1	0
12	22.00-24.00	0	2	0	0	0	0
13	24.00-26.00	0	0	0	0	0	0
14	26.00-28.00	1	0	0	0	0	0
15	28.00-30.00	0	0	0	0	0	1
Total number		114	77	14	50	64	8
Mean diameter		10.45	9.71	8.25	10.20	10.27	11.00
SD		3.42	3.45	2.57	4.49	4.34	7.46
Min.		5.00	5.00	4.00	5.00	4.00	6.00
Max.		26.00	19.00	14.00	21.00	20.00	28.00

experimental monkeys, especially in the Ca, Mg-deficient monkey (Ex-1a, b), the number of argyrophilic bodies was increased in both cervical and lumbar anterior horns as compared with the control monkeys (C-1a, b). The longitudinal diameter of the body ranged from 4 to 18 μm with the mean value of about 10 μm . The bodies were distributed in the ventral border of anterior horns (Fig. 1A). A large argyrophilic body with a diameter of 18 μm was found beside the motor neuron in the dorsolateral nucleus of anterior horn in the Ca, Mg-deficient and Al surplus monkey (Ex-2a) (Fig. 1B). In all groups, there were a few bodies with a diameter of over 20 μm , whose frequency did not differ among the experimental groups.

Morphometry of Cellular and Nuclear Surface Area

The total number of neurons examined in each monkey was 168 (Ex-1a), 125 (Ex-1b), 117 (Ex-2a), 210 (Ex-2b), 129 (Ex-3), 94 (C-1a) and 77 (C-1b) per section. The total number of neurons in the two control monkeys was rather small as compared with that of experimental monkeys. Also, there was no statistically significant difference in the total number

between the two control monkeys. Therefore, the data in control animals were mixed and treated as one group ($n=171$).

The morphometric findings of cellular surface area of lumbar spinal neurons are shown in Fig. 2. In the control group, three peaks are seen, which correspond to small, intermediate and large sized neurons. In sharp contrast, skewed unimodal distribution is found in the Ca, Mg-deficient (Ex-1a, b) and Ca, Mg-deficient and Al surplus (Ex-2a, b) groups, in which only the peaks for small sized neurons could be observed (Kolmogorov-Smirnov two sample test, $P<0.05$ and $P<0.01$, respectively). Particularly in the latter group, a marked relative decrease in the number of large sized neurons (over 400 μm^2) was seen. The histogram of the Al excess group (Ex-3) was, however, not different from that of the control group.

The results of the morphometric analyses of nuclear surface area of lumbar motor neurons are shown in Fig. 3. The Ca, Mg-deficient and Ca, Mg-deficient and Al surplus groups exhibited a unimodal distribution, indicating a shrinkage of nucleus over 60 μm^2 in size; in the latter group, the difference between the control is significant at $P<0.01$. But the histogram for the Al excess group showed a bi-

Fig. 1 A: Argyrophilic bodies (arrows) in the ventral border of lumbar anterior horn of a Ca,Mg-deficient and Al surplus monkey (Ex-2a). $\times 250$. B: Large argyrophilic body (arrow) with a diameter of $18\text{ }\mu\text{m}$ found beside a motor neuron in dorsolateral nucleus in the lumbar anterior horn of the same monkey (Ex-2a). $\times 500$

modal pattern similar to that for the control group.

Nuclear and cellular surface areas are highly correlated in the control and experimental

groups ($r=0.85863\text{--}0.926646$, $P<0.001$) as shown in Fig. 4. The neurons in the Ca,Mg-deficient and Ca,Mg-deficient and Al surplus groups were, however, densely distributed in

Fig. 2 Histograms of cellular surface area of lumbar spinal neurons. Probabilities (p) were evaluated with Kolmogorov-Smirnov two sample test.

Fig. 3 Histograms of nuclear surface area of lumbar spinal neurons. Probabilities (p) were evaluated with Kolmogorov-Smirnov two sample test.

the smaller sized region with respect to both nuclear and cellular surface areas. In the control and Al excess groups, these areas show a more diverse distribution at a 95% confidence level.

Neuropathologically, simply atrophic neurons and chromatolytic neurons with the nucleus dislocated to the periphery of the perikaryon were frequently found in the experimental monkeys, particularly in the Ca,Mg-deficient and Al surplus group (Fig. 5).

DISCUSSION

Monkeys in the Ca,Mg-deficient and Al surplus group revealed an axonal swelling filled with compactly arranged 10-nm neurofilaments and chromatolytic neuronal changes in the anterior horn of spinal cord; Al content in the brain tissue was slightly increased, although not significantly (13). These findings are comparable with changes in anterior horn cells at early stages of ALS, especially in a case with an unusually short clinical course (5, 6). Carpenter (1) reappraised argentophilic spheroids in the proximal portions of axons of anterior horn cells in patients with ALS, and distinguished between larger (over 20 μm) spheroids and other argentophilic structures, the so-called globules. The latter is known to be present in a variety of cases including the normal individual, but is ultrastructurally similar to those found in the ALS case (10). The present morphometric study showed an

increase in the amount of globules to a various extent among the experimental groups, occasionally with large argentophilic bodies over 20 μm (Tables 2 and 3). The globules were usually distributed in the ventral border of anterior horn, but sometimes found beside a motor neuron in the dorsolateral nuclei of spinal cord in experimental monkeys (Fig. 1).

In morphometry of cellular and nuclear surface areas, is shown a relative decrease in the number of large sized neurons in the spinal cord of monkeys fed on the Ca,Mg-deficient diet without or with supplemental aluminum (Fig. 2). Under a Ca,Mg-deficient condition, supplemental Al seems to potentiate the development of axonal swelling, simple atrophy or shrinkage, and chromatolysis with dislocation of the nucleus. Results in the present study, however, failed to reveal an absolute decrease in the number of large sized neurons, and it is still unclear if a relative decrease in the number of large sized neurons results from a shrinkage of motor neurons or neuronal cell loss. On the other hand, the morphometric findings in monkeys fed on the Al excess diet were not different from those in control monkeys (Fig. 2).

In this study, motor neuron degeneration was accelerated by a Ca,Mg-deficient diet without or with supplemental Al, especially by the latter, but was not accelerated by Al excess only. These experimental conditions simulate unusual environmental features common to all three high-incidence foci of ALS in the

Fig. 4 Correlation between the cellular surface area and nuclear surface area of lumbar spinal neurons

Western Pacific (3). Recent epidemiological studies in these foci have increasingly indicated the importance of environmental factors in the occurrence of ALS (2, 8, 9, 12). Analyses of environmental specimens, including soil and water samples from the Guam and Kii foci, have shown a high content of Mn and Al, and a low content or lack of Ca and Mg (14,

15). Employing neutron activation analysis, Yoshimasu *et al.* (21) found high contents of Al and Ca in the central nervous system (CNS) tissue of patients with ALS or Parkinsonism-dementia complex (PDC), with significant correlations between Ca and Al, and/or between Ca and Mn. Furthermore, the coprecipitation of Ca and other di- or trivalent

Fig. 5 A chromatolytic neuron (thick arrow) and simply atrophic neurons (thin arrows) in the lumbar anterior horn of the Ca,Mg-deficient and Al surplus monkey (Ex-2a). $\times 250$

metals such as Mn and Al were confirmed by X-ray microanalysis in the degenerative lesions of cervical spinal cord of ALS patients (17–19), and the product was identified as Ca-hydroxyapatite (7, 16, 20). It is thus possible that the pathogenetic process of ALS or PDC in patients in the Kii and Guam foci is related to a geological and geochemical condition, *i.e.*, a prolonged deficient intake of Ca and Mg together with excess exposure to Al and other toxic minerals.

The present study was undertaken to evaluate the above possibility, which is our working hypothesis. Yase (15) described a metal-induced calcifying degeneration of motor neurons in primates. There still remain several problems. First, our results failed to exhibit a significant increase in Ca and Al content in the CNS tissue of experimental monkeys. Second, absolute neuronal loss in the spinal cord has not been induced in experimental monkeys. Third, inclusion bodies specifically associated with ALS, such as the Bunina body and others, have not been found in spinal neurons of experimental monkeys. Garruto *et al.* (4) recently reported basophilic inclusions, considered to be 'the most signifi-

cant and perhaps pathognomonic phenomenon in ALS', in spinal neurons of cynomolgus monkeys fed on low-Ca, high-Al diet for a longer period than that in our study. Further detailed investigation is required in relation to age of monkeys, dietary conditions, feeding periods, and chemical forms of supplemental aluminum.

The authors thank the members of the Department of Neurophysiology, Primate Institute of Kyoto University, for their kind cooperation. We also thank Junko Yamamoto and Katsumi Furuto for their devoted technical assistance and Dorothy Grier, R. N. of the Division of Neurological Diseases for reviewing the manuscript.

Received for publication 18 October 1989; and in revised form 6 November 1989

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