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Fragmentation of the Golgi Apparatus of Motor Neurons in Amyotrophic Lateral Sclerosis (ALS)

Clinical Studies in ALS of Guam and Experimental Studies in Deafferented Neurons and in β,β′-Iminodipropionitrile Axonopathy

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Previous morphological immunoenzymatic studies with organelle-specific antibodies have disclosed an apparent fragmentation of the Golgi apparatus in large numbers of motor neurons in 12 cases of sporadic, non-Guamanian amyotrophic lateral sclerosis (ALS) in three cases of other types of motor neuron disease and in one case of a mitochondrial myopathy with cytochrome c oxidase deficiency. Motor neurons with fragmented Golgi apparatus were moderately atrophic; in these cells, discrete immunostained elements of the organelle were twice as many as in normal neurons, and the size of each Golgi element and the percentage of the cytoplasmic area occupied by the Golgi apparatus were reduced (Am J Pathol 1992, 140: 731–737). In this report we have confirmed the fragmentation of the organelle of motor neurons in the spinal cord in six sporadic cases of Guamanian ALS. In four of the six cases the clinical course was 1 to 2 years. The percentages of motor neurons with fragmented Golgi apparatus varied from 38 to 92. Motor neurons from three additional cases of Guamanian ALS of clinical duration from 5 to 7 years did not show fragmentation of the Golgi apparatus. In two cases of Guamanian ALS and in one non-Guamanian ALS, all neurons with ubiquitin-positive skein-like or granular inclusions believed to be pathognomonic for ALS had fragmented Golgi apparatus. To examine whether the fragmentation of the Golgi apparatus results from reactions to either neuronal deafferentation or to lesions of proximal axons, we conducted two experimental studies. In the first study, we examined in cats the Golgi apparatus of deafferented neurons of the dorsal lateral geniculate nucleus. In the second study, we examined the Golgi apparatus of motor neurons in the spinal cord of rats with proximal axonopathy induced by β,β′-iminodipropionitrile. In these two experiments, the neuronal Golgi apparatus studied by immunoenzymatic techniques and morphometry, was not fragmented. Taken together, the results of these studies strongly suggest that the fragmentation of the Golgi apparatus of motor neurons in ALS represents an important and perhaps early change of the organelle that may be involved in the pathogenesis of ALS. The fragmentation of the Golgi apparatus of motor neurons is a fairly specific and easily recognizable marker of ALS and may be used together with other criteria for comparisons between the human disease and proposed animal models of the disorder. (Am J Pathol 1994, 144: 1288–1300)

Numerous studies have emphasized the importance of the Golgi apparatus in a host of postranslational modifications of polypeptides destined for secretion and plasma membrane synthesis and in the targeting

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of lysosomal enzymes.[1] In neurons, the Golgi apparatus is involved in centripetal membrane traffics, transneuronal transport of exogenous ligands, and orthograde fast axonal transport.[2–4] Therefore, it is reasonable to speculate that an abnormality of this key organelle will have serious functional implications.

Until recently, morphological studies of the Golgi apparatus in human pathological tissues obtained at autopsy were either difficult to perform or not feasible. This difficulty has been circumvented with the immunoenzymatic detection by highly specific antibodies of an antigen of the Golgi apparatus that is relatively resistant to autolytic changes.[5–7] These reagents have been applied to morphological studies of the Golgi apparatus in experimentally induced chromatolysis and in amyotrophic lateral sclerosis (ALS) and related diseases.[6,8–10]

We have reported that the Golgi apparatus appears to be fragmented in a high percentage of spinal cord motor neurons in ALS and other related motor neuron diseases.[8–10] This fragmentation of the organelle has also been observed in one case of mitochondrial myopathy due to a deficiency of cytochrome C oxidase.[10] In normal spinal cord motor neurons, the immunostained Golgi apparatus is in the form of a network of irregular granular or linear densities that fill the perikaryon and extend into proximal segments of dendrites. In contrast, in neurons with a fragmented Golgi apparatus, the immunostained elements of the organelle in perikarya and dendrites are smaller, discrete, and more numerous than in normal neurons. Morphometric studies have confirmed the qualitative observations.[9] The resolution of the light microscope is not sufficient to establish whether the smaller and discrete elements of the fragmented Golgi apparatus are indeed separate or interconnected.

Histopathological studies in sporadic, familial, and Guam cases of ALS suggest that ubiquitin-positive skein-like inclusions in motor neurons are characteristic of various forms of ALS.[11,12] To gain additional insights into the significance of the fragmentation of the Golgi apparatus of motor neurons in ALS, we conducted the clinical part of this study to answer two questions, ie, whether 1) the fragmentation of the organelle occurs in Guam ALS and 2) there is correlation between the ubiquitin inclusions and the fragmentation of the Golgi apparatus.

For numerous reasons, the interpretation of morphological abnormalities of the Golgi apparatus of motor neurons in tissues obtained at autopsy and detected by immunoenzymatic methods is problematic. Artefacts due to postmortem autolysis must be excluded, previous experimental manipulations of the tissues are not possible, and the assessment of the developmental history of the lesion is not possible. Specifically, in ALS, at least two questions concerning the significance of the observed fragmentation of the Golgi apparatus may be asked. First, does this change reflect a general reaction to an axonal lesion? In ALS, axonal swellings or spheroids have been detected with classical histological methods and with the immunoenzymatic detection of neurofilament epitopes.[13] Second, is the fragmentation of the Golgi apparatus of motor neurons in the spinal cord due to the extensive degeneration of the corticospinal tracts and deafferentation of those neurons?[14]

We have attempted to probe the first question, ie, whether the fragmentation of the Golgi apparatus of motor neurons is associated with an injury of proximal segments of axons, by investigating the effect of β,β'-iminodipropionitrile (IDPN) in rat motor neurons. Since the original paper by Chou and Hartmann, the proximal axonopathy induced by IDPN has been extensively studied.[15–18] In the proximal segments of axons of rats treated with IDPN, there is a remarkable redistribution of neurofilaments that are found in the periphery of axons, whereas microtubules, smooth endoplasmic reticulum, and mitochondria are found in the center of axons.[16] In IDPN-induced axonopathy the rate of the fast component of axonal transport is not changed, whereas the slow transport of neurofilament proteins is impaired.[16,17] More importantly, with the possible exception of axonal spheroids, the clinical and histopathological pictures of the IDPN-induced axonopathy bear no relationship to motor neuron disease in humans therefore, this experimental paradigm is appropriate to investigate whether the Golgi fragmentation may be associated with other types of axonal injury.

We have attempted to probe the second question, ie, whether the fragmentation of the Golgi apparatus of motor neurons in ALS results from deafferentation, by examining in cats the effect of eye enucleation on the Golgi apparatus of neurons in the dorsal lateral geniculate nucleus (LGN). In this animal, the projection of crossed and uncrossed fibers from the retina to the LGN and the pattern of atrophy of neurons of the LGN after retinal lesions or enucleation of the eye have been extensively studied.[19–21]

Materials and Methods

Clinical Studies

Guam ALS

Nine cases of Guam ALS, examined and autopsied by one of us (AH), were studied and summarized in Table 1. On neuropathological examination, all cases showed typical ALS lesions. Three more Guamanian

patients with advanced ALS were also studied but are not included in this paper because of insufficient numbers of surviving motor neurons.

Controls

In previous studies age-matched and selected neurological controls of approximately equal numbers to the ALS cases were used.[8–10] In the previous controls, the percentages of spinal cord motor neurons with a fragmented Golgi apparatus ranging from 0 to 2.8 were significantly lower than those in ALS that ranged from 8.3 to 59. In this study we used two controls to rule out a possible effect of postmortem autolysis on the fragmentation of the Golgi apparatus.

The first control (case 93–133) was a 58-year-old woman with diffuse atherosclerosis who died with a massive infarction of the small and large intestines. The body was kept at 4 C and autopsy was performed 74 hours later. On gross examination there were no abnormalities in the brain and spinal cord. Microscopic examination of sections from brain and spinal cord revealed palor of neurons and retraction of the neuropile around neurons consistent with autolytic changes.

The second control (case 93–151) was a 73-year-old woman with poorly controlled hypertension who died from sepsis and renal failure secondary to an aortic graft infection. The brain and spinal cord, examined 6 hours after death, were normal on gross and microscopic examinations. Cross-sections from the cervical, thoracic, and lumbar segments of the spinal cord were taken, placed in buffered physiologic saline (phosphate-buffered saline [PBS]), pH 7.4, and stored at room temperature for 24, 48, and 115 hours. To avoid the growth of bacteria or fungi, the PBS solution contained 0.02% sodium azide. At each of the above time points, sections were fixed in neutral-buffered formalin for 48 hours and embedded in paraffin.

Experiments with IDPN

Eight female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200 to 300 g were used in this study. IDPN (Eastman Kodak, Rochester, NY) was diluted 1:5 in saline and the pH adjusted to 7.4 with hydrochloric acid. Rats that were allowed to survive for less than 10 days received one intraperitoneal injection of IDPN (2 mg/g body weight), whereas those killed at longer time intervals received the same total dose divided in three equal injections given intraperitonealy every fourth day. One rat used as control received an equal volume of saline. The animals were checked every 30 minutes after the injection; they did not show any signs of pain and were returned to the colony when they were able to eat and drink on their own. Hyperexcitability, circling, and choreiform movements (the ECC syndrome so named for excitement, circling, and choreiform movements) appeared after 24 hours in the rats that received the full dose of IDPN in one injection. A milder form of this syndrome developed after the first injection in rats that received the same total dose of IDPN in three injections; the full ECC syndrome developed after the final injection of IDPN.

Tissue Processing

The control rat and one rat sacrificed 10 days after one injection of IDPN were anesthetized in ether and perfused via the left ventricle for about 10 minutes with a solution of 4% paraformaldehyde (EM Sciences, Fort Washington, PA) in 100 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl (PBS). The lumbar segment of the spinal cord and segments of the sciatic nerve, proximal to its trifurcation, were sampled and immersed in the same fixative for 5 to 6 hours at room temperature. The rats sacrificed 1, 3, and 7 days and 2, 3, and 16 weeks after administration of IDPN were anesthetized and killed in a chamber containing dry ice. The lumbar segment of spinal cord of these animals was removed and immersed immediately in 1% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4, for 5 to 6 hours at room temperature. All tissues were then washed three times for 15 minutes in 50 mmol/L ammonium chloride in PBS and once in PBS for 15 minutes. The tissues were then dehydrated, infiltrated with paraffin in an Autotechnicon Mono (Technicon Industrial Systems, Tarrytown, NY), and embedded in paraffin. Cross-sections 5-μ thick were cut and mounted on poly-L-lysine-coated glass slides.

Deafferentation Experiments

Eye Enucleation

Four domestic short hair female young adult cats (between 1 and 5 years old) purchased from Liberty Farms were used in this study. Under general anesthesia, induced and maintained by acepromazine (0.05 to 0.11 mg/kg) intramuscularly and inhalation of halothane (1 to 3%) and after the injection of 0.02 mg/kg of atropine sulfate sq. and of procaine penicillin (20,000 U/kg), enucleation of the right eye was performed using aseptic surgical techniques. Briefly, the globe was exposed, the conjuctiva, Tenon's capsule, and extraocular muscles were incised and dissected from their attachment to the globe and the optic nerve was clamped and then cut with curved

enucleation scissors. Gelfoam was packed against the posterior orbital surface, the membrane nictitans was removed, and the lid margins were sutured. The remaining conjuctiva and Tenon's capsule portion were closed with 3-0 cat gut. The lid margins were sutured using a 4-0 nylon or silk. After the operation, the animals were placed on a circulating warm water heating pad and checked every 15 to 30 minutes. The animals were returned to the colony when able to thermoregulate, eat, drink, and stand on their own. The animals were checked twice daily for discomfort, pain, or infection until sacrifice. The recovery of all animals was uneventful and there was no need for sedation or antibiotic treatment.

Tissue Processing

The four cats were sacrificed after 1, 4, 8, and 9 months after the enucleation. Under deep barbiturate anesthesia (pentobarbital, 60 mg/kg intravenously) the descending aorta was clamped and the animals were perfused via left ventricular puncture with 2 L of PBS, pH 7.4, followed by 2 L of 1% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4. The LGNs were removed immediately and immersed in the same fixative for 5 hours. The tissue was then washed three times for 15 minutes each time with 50 mmol/L ammonium chloride in PBS and once again for 15 minutes in PBS. Dehydration, clearing, and paraffin-infiltration were then conducted on an Autotechnicon Mono. The tissues were paraffin embedded, cross-sectioned serially at 5 μ, and mounted on poly-L-lysine-coated slides.

Antibodies

As a marker for the Golgi apparatus, in human and cat tissues, a rabbit polyclonal anti-serum against MG-160, a sialoglycoprotein of the medial cisternae of the organelle, was used at 1:1000 dilution. In rat tissues, undiluted monoclonal antibody 10A8 against the same protein was used.[5,7]

Monoclonal antibody TA51 against the phosphorylated NFH and NFM neurofilament subunits was a gift from Dr. W.W. Schlaepfer[22] and was used at 1:50 dilution. A rabbit polyclonal anti-serum against ubiquitin was obtained from Dako (Carpinteria, CA) and used at 1:1000 dilution.

Immunoenzymatic Methods

The slides were dehydrated and permeabilized either with 0.5% saponin and 3% fish gelatin in PBS or with 0.5% Triton X-100 in PBS for 15 to 20 minutes and then blocked in 3% fish gelatin in PBS or in 10% horse serum in PBS, respectively. The slides were then incubated overnight with the primary antibody, the next day slides were washed in PBS and incubated with either biotinylated horse anti-rabbit IgG (Vector, Burlingame CA) if the primary antibody was from rabbit or with biotinylated horse anti-mouse IgG (Vector) if the primary antibody was from mouse, followed by incubation in an avidin-biotin peroxidase conjugate (Vector).[23] Peroxidase reaction was revealed either with the method of Graham and Karnovsky[24] with diaminobenzidine tetrahydrochloride as substrate or with Vector-VIP obtained from Vector.

Slides with sections of spinal cord from human tissues that were fixed in formalin and embedded in paraffin were microwaved after rehydration but before permeabilization according to the method of Shi et al[25] to retrieve the antigenic epitopes. This method was applied in a previous published study on ALS.[10] Briefly, after deparaffinization slides were immersed in a glass jar containing 1% zinc sulfate in distilled water, the jar was placed in a microwave oven, and the water brought to boil twice for 5 minutes each time. Slides were then permeabilized and processed for immunostaining as described above.

Morphometry and Statistical Methodology

Morphometric analyses of mean surface areas of entire cell, cytoplasm, nucleus, and Golgi apparatus were conducted, as reported previously, with a CUE-2 image analyzer using the planomorphometry program (Olympus, Lake Success, NY).[9] Additional measurements included numbers of immunostained Golgi profiles per cell and percentages of cell surface areas occupied by the Golgi apparatus. The statistical significance of the obtained values were evaluated by Student's *t*-tests performed between the values obtained in control and test animals. To avoid counting a section from the same cell twice, serial 5-μ thick sections were obtained but only every 10th section was analyzed by morphometry.

Results

Clinical Studies

In Table 1, the age, sex, and duration of illness of the Guam patients with ALS studied are summarized. Unlike the sporadic non-Guam cases of ALS previously reported from this laboratory in the Guamanian ALS the onset of the disease was observed in younger

Table 1. *Guamanian Patients with ALS Controls*

Guam ALS	Age at Death	Sex	Duration of Illness (*in Years*)	No. of Neurons Counted	% Fragmentation
AGUON	41	M	2	341	57
GA 82–64	54	M	5	340	1.2
GA 33–64	52	M	6	51	92
BABAUTA	34	F	5	43	0
GA 152–65*	32	F	7	70	0
GA 47–64	58	M	1	183	85
GA 149–64	54	M	2	98	67
GA 142–65	57	M	2	26	38
GA 122–65	62	M	5	129	76

Controls	Age	Sex	Cause of Death	No. of Neurons Counted	% Fragmentation
93–133	58	F	Extensive atherosclerosis massive infarction of small and large intestine	148	3.3
93–151	73	F	Hypertension sepsis and renal failure secondary to an aortic graft infection	160	4.3

* Familial case.

individuals, whereas the duration of ALS was approximately similar.[8–10] One of the nine cases studied, (GA 152-65) was familial, because her father and two sisters had ALS.

Also in Table 1, the average numbers of spinal cord neurons with normal or fragmented Golgi apparatus of both Guam ALS cases and controls counted independently by two of us (ZM and NKG) are presented. The results of these two independent counts were so close that the average figures provide a fairly accurate estimate of the frequency of fragmented Golgi apparatus. With the exception of case GA 33–64, the highest numbers of motor neurons with fragmented Golgi apparatus were found in cases with a brief duration of the disease. Most neurons with fragmented Golgi apparatus were found in sections of the lumbar cord, perhaps because this level of the cord was relatively spared in the cases studied and therefore the chances to observe well preserved neurons were higher. Also, this finding suggests that fragmentation of the Golgi apparatus occurs relatively early during the course of neuronal degeneration.

In Figure 1a, the typical fragmentation of the Golgi apparatus of a motor neuron and the usual pattern of the Golgi apparatus in an adjacent normal neuron are displayed. The fragmented Golgi apparatus appears in the form of numerous, small immunostained round densities found throughout the perikaryon, and in proximal and distal dendrites. In contrast, the usual pattern of the Golgi apparatus of motor neurons is in the form of irregular immunostained densities, also found in the perikaryon and dendrites. However, in normal motor neurons, these usual Golgi profiles are fewer, larger, and less regular in their outlines than the elements of the fragmented Golgi apparatus. Previous morphometric studies on the Golgi apparatus of motor neurons in ALS are consistent with the qualitative analyses.[8,9]

Figure 1b of spinal cord motor neurons, taken from the control patient whose autopsy was performed 74 hours after death, shows normal immunostain pattern of the Golgi apparatus. The intensity of the immunostained Golgi membrane protein and the profiles of individual Golgi elements are virtually identical to those found in patients autopsied within 24 hours from death.[8–10] A similar normal immunostain of the Golgi apparatus was obtained from spinal cord sections stored in PBS at room temperature for 24, 48, and 115 hours before fixation and immunostaining (data not shown). Therefore, the antigenic determinants of MG-160, the intrinsic membrane sialoglycoprotein of the Golgi apparatus detected by the polyclonal antiserum, are resistant to postmortem autolysis and the described fragmentation of the organelle is not due to postmortem effects.

In Figure 2, taken from a section of the spinal cord of a patient with Guam ALS, one motor neuron with a normal immunostained Golgi apparatus is surrounded by neurons with fragmented organelle.

To investigate possible correlations between ubiquitin-stained inclusions in ALS motor neurons and the state of the Golgi apparatus, serial 5 μ sections were stained alternatively with anti-sera against MG-160 or ubiquitin. Double staining for these two antigens was attempted but the results were not satisfactory. Figure 3a, is a low magnification photomi-

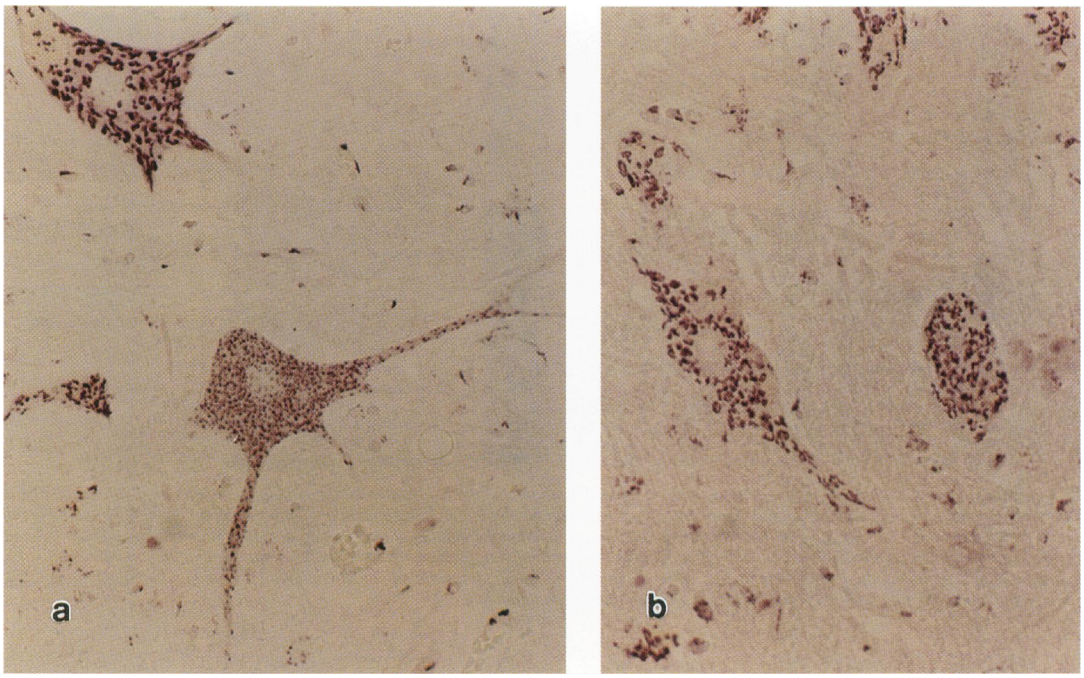

Figure 1. a: *ALS. Two spinal motor neurons immunostained with a polyclonal anti-serum against MG-160, an intrinsic membrane protein marker of the Golgi apparatus. In the center of figure, neuron with fragmented Golgi apparatus represented by small immunostained elements of the organelle in perikaryon and dendrites. In the upper part of the figure, neuron with the usual profiles of the Golgi apparatus in the perikaryon and dendrites* b: *Control, (autopsied 74 hours after death), stained as in* a. *Note usual profiles of the Golgi apparatus (×1280).*

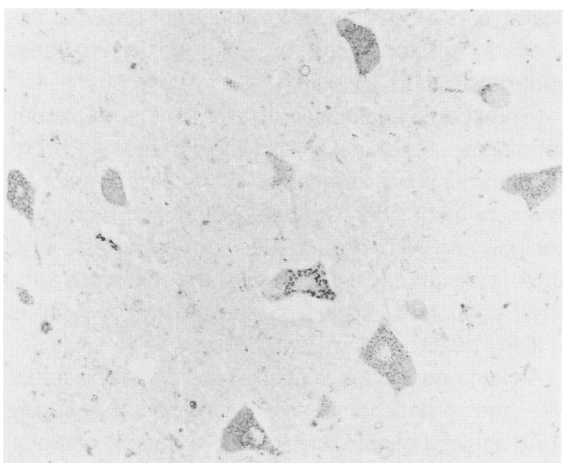

Figure 2. *ALS. Several spinal cord motor neurons immunostained as in Figure 1. In center, neuron with usual profiles of the Golgi apparatus surrounded by neurons with fragmented organelle (×700).*

crograph of a small group of motor neurons stained for the Golgi apparatus. Figure 3b, illustrates the immediately consecutive section of the spinal cord stained for ubiquitin. Inserts in Figure 3, a and b are taken at a higher magnification of fields from Figure 3, a and b to illustrate the Golgi apparatus in two neurons with ubiquitin-positive inclusions. The Golgi apparatus in both neurons is indeed fragmented. However, the ubiquitin-positive inclusions do not contain elements of the Golgi apparatus, as revealed by the immunostain for MG-160 (compare in inserts in Figure 3, a and b, neuron indicated by arrowhead). To determine the degree of correlation between ubiquitin-positive inclusions and fragmented Golgi apparatus, we analyzed three ALS cases that contained the highest number of ubiquitin-positive inclusions (Table 2). The results of this analysis strongly suggest that the ubiquitin-positive inclusions develop in neurons with fragmented Golgi apparatus. This analysis has also revealed a significant number of ghost-like neurons with ubiquitin inclusions in which there was virtually no stainable Golgi apparatus.

IDPN Experiment

Rats that received the full dose of IDPN in one injection developed within 24 hours the typical ECC syndrome, consisting of excitement (hyperexcitability), random circling, choreiform movements of the head, and tremors.

Rats that received the same total IDPN dose in three injections developed 24 hours after the first injection a milder form of the syndrome that consisted mainly of hyperexcitability; these animals displayed less circling or choreiform head movements. In those animals the full ECC syndrome was observed after the

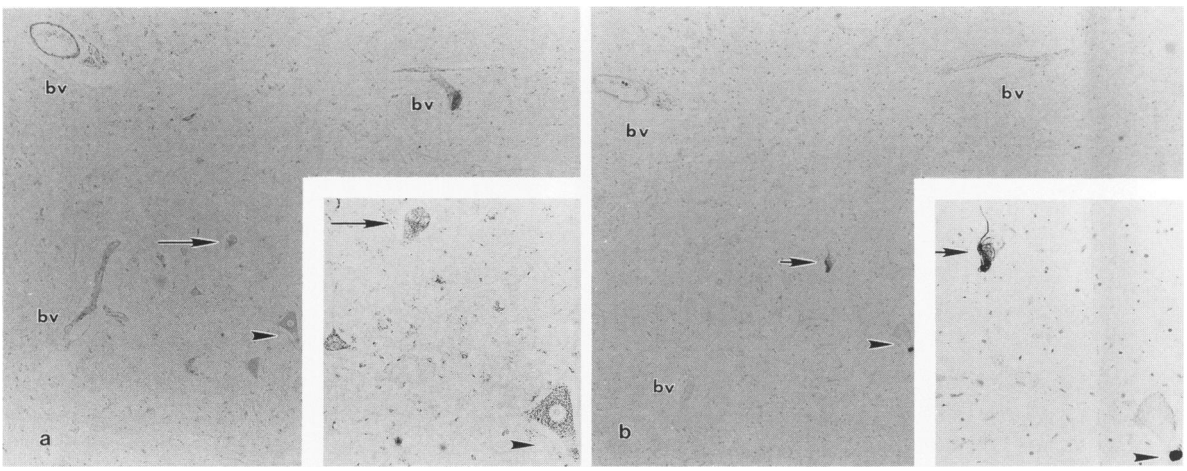

Figure 3. a: *Motor neurons from section of spinal cord from an ALS patient stained for the Golgi apparatus as in Figures 1 and 2.* b: *Consecutive section stained with antibody against ubiquitin; for orientation see blood vessels (bv). Arrows indicate same neurons stained for the Golgi apparatus in* a *and ubiquitin in* b (×200). *Inserts: Higher magnifications:* a *stained for the Golgi apparatus; arrow and arrowhead show two neurons, which on* b *show ubiquitin-positive skein-like inclusions* (arrow) *or a granular inclusion* (arrowhead). *Note that both neurons with ubiquitin-positive inclusions have fragmented Golgi apparatus. Also, note that in neuron indicated by arrowhead, the area with a granular ubiquitin-positive inclusion is not stained with the antibody against the Golgi apparatus* (×720).

Table 2. *Correlation Between Ubiquitin Inclusions and Fragmentation of the Golgi Apparatus*

	No. of Neurons	Neurons with Normal Golgi	Neurons with Fragmented Golgi/ (Ubiquitin-Positive Neurons)*
Case			
92–195†	107	46	61/(19)
AGUON	147	27	120/(29)
GA 33–64	87	37	50/(21)

* In parentheses are numbers of neurons with ubiquitin inclusions.
† Case reported previously (Ref. 10).

final injection of IDPN. One rat sacrificed 16 weeks after the initial administration of IDPN showed a gradual but complete recovery from the ECC syndrome and was virtually asymptomatic during the last 4 weeks of life.

Cross-sections from the sciatic nerves of the IDPN-treated rats immunostained with a monoclonal antibody against the phosphorylated NFH and NFM neurofilament subunits disclosed the typical displacement of the neurofilaments to the periphery of axons, whereas the central area of axons was devoid of neurofilaments (Figure 4a).[16] In contrast to the rat treated with IDPN, the control rat showed in sciatic nerve axons a uniform distribution of the immunoreactivity for neurofilaments (Figure 4b). Sections of the spinal cord of IDPN-treated rats stained with the Bodian's method showed the classical proximal axonopathy characterized by enlarged nerve fibers and roots (Figure 5).[15–18]

Microscopic examination of sections from lumbar segments of the spinal cord of IDPN-treated and control rats stained with hematoxylin and eosin disclosed motor neurons with central nuclei and prominent nucleoli and with Nissl bodies of normal size and amount distributed throughout the perikaryon; atrophy, degenerative changes, or loss of motor neurons in IDPN-treated rats were not observed.

An immunoenzymatic stain of the Golgi apparatus with the organelle-specific monoclonal antibody 10A8 showed no difference of the organelle of motor neurons between IDPN-treated and control animals (Figure 6, a and b). The Golgi apparatus appeared to be intact with the usual large irregular ovoid or spherical and often interconnected Golgi profiles, occupied

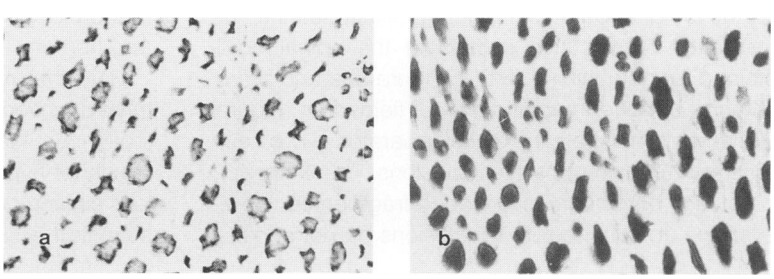

Figure 4. a: *Cross-section of sciatic nerve from rat given IDPN once 10 days before sacrifice and stained with antibody against phosphorylated epitopes for neurofilament proteins (NF). Note peripheral distribution of NF proteins.* b: *Cross-section of sciatic nerve from control rat, stained as in Figure 5. Note even distribution of NF immunoreactivity* (×4200).

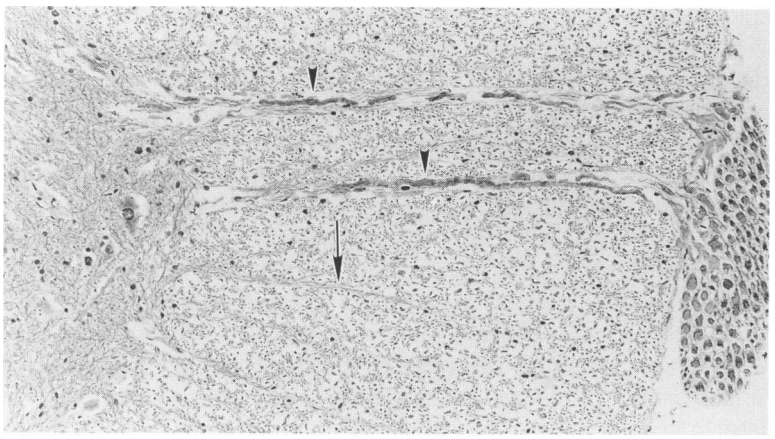

Figure 5. *Spinal cord section from same rat as in Figure 4a but stained for axis cylinders according to Bodian's method. Note swelling of two proximal axons* (arrowheads) *cut along the longitudinal axis; compare with normal axons* (arrow) (×650).

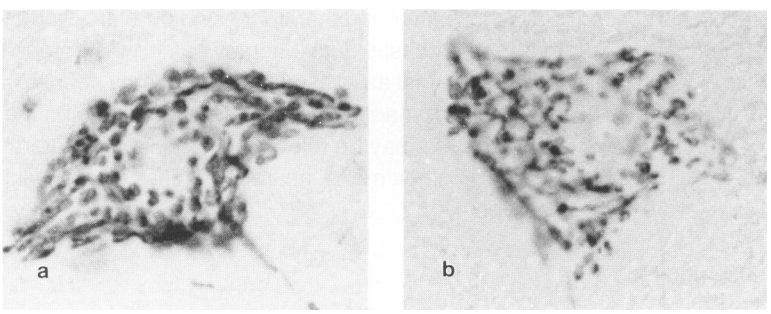

Figure 6. a: *Spinal cord motor neuron from same rat treated with IDPN as in Figure 4a immunostained for the Golgi apparatus.* b: *Spinal cord motor neurons from control animal stained for the Golgi apparatus. The Golgi apparatus of these motor neurons is similar; qualitative morphological observations were confirmed by morphometric studies (see Table 3)* (×3200).

a perinuclear zone, and extended to proximal dendrites but not in axons (Figure 6, a and b). There was no apparent reduction of the area occupied by the Golgi apparatus or fragmentation of the organelle in the motor neurons in control or IDPN-treated animals.

To further investigate the possibility of subtle changes of the Golgi apparatus of motor neurons in IDPN-treated animals, we examined by a morphometric method 20 motor neurons from lumbar segments from each of two animals sacrificed 10 days and 16 weeks after IDPN injections and one control animal. The results, summarized in Table 3, do not show significant differences between the one control and the two IDPN-treated animals in all parameters measured, ie, the means of cell, cytoplasmic, nuclear, and Golgi areas, and the means of numbers of stained Golgi apparatus per cell and percentages of total cell area occupied by the organelle.

Deafferentation Experiment

The projection of crossed and uncrossed fibers from the retina to the LGN and the pattern of atrophy of the neurons of the LGN after retinal lesions or enucleation of the eye in the cat has been extensively studied.[19–21] The right eye from the four cats was surgically removed and atrophy of the neurons in layer A1 from the ipsilateral LGN and atrophy of layer A from the contralateral LGN was observed. The atrophy was mild in animals sacrificed 1 and 2 months after enucleation and very prominent in the two animals sacrificed 8 and 9 months after enucleation.

Table 3. *Morphometric Studies in IDPN-Treated and Control Rats*

	Control	IDPN—10 days	IDPN—16 weeks	*P*
Mean cell area/μ²	1000.6 ± 233.7	996.4 ± 169.7	953 ± 211.8	NS
Mean cytoplasmic area/μ²	890.2 ± 197.1	898.5 ± 151.6	865.5 ± 198.6	NS
Mean nuclear area/μ²	110.4 ± 49.9	97.94 ± 29.7	87.5 ± 21.9	NS
Golgi/size/μ²	5.1 ± 1.59	6.3 ± 2.1	5.2 ± 1	NS
Golgi elements/cell	36.7 ± 7.5	33.6 ± 8.1	32.7 ± 4.2	NS
% Golgi/cell	19.2 ± 2.4	20.7 ± 4.1	18.3 ± 3.2	NS

P values are based on the Student's *t*-test. NS, not significant. Twenty motor neurons were analyzed from each IDPN-treated animal and 20 from the control. Five-micron thick sections were analyzed and to avoid analyzing the same cell twice; of the serial sections only every 10th was used in the study.

Atrophic neurons showed smaller cytoplasmic and nuclear areas and an apparent reduction of the Nissl substance (Figure 7); there was no evidence of degeneration of atrophic neurons. In atrophic neurons the Golgi apparatus was not fragmented (Figure 8). Morphometric studies performed in both atrophic and normal LGN neurons, summarized in Table 4, showed that in atrophic neurons mean cell, nuclear, and cytoplasmic areas were significantly reduced. Also, in atrophic neurons, the numbers of Golgi elements per cell and surface areas occupied by a single Golgi element were significantly reduced. However, in atrophic neurons the percentages of total surface areas occupied by the Golgi apparatus were similar to those of normal neurons (Table 4). These results enhance the conclusion that in ALS the fragmentation of the Golgi apparatus of motor neurons is not due to atrophy caused by deafferentation.

Discussion

IDPN Experiment

In rats, IDPN induced the typical clinical symptoms and histopathological lesions in proximal axons.[15–18] Despite the massive rearrangements of cytoskeletal elements in proximal axons induced by IDPN, in neuronal perikarya there were no structural changes of the Golgi apparatus (Figures 4, a and b, 6, a and b, Table 3).[16] Most likely the IDPN-induced separation of microtubules from neurofilaments in axons is not associated with a similar massive rearrangement of microtubules and neurofilaments in the adjacent perikaryon. The published observations that fast components of axoplasmic transport are not affected by IDPN, whereas components of the slow axoplasmic transport are affected by IDPN, suggest that the functions of the Golgi apparatus, a key organelle for the processing of glycoproteins destined for fast axoplasmic transports, are not affected by IDPN.[4,16,17]

The relationships between experimental or clinical axonic neuropathies and the Golgi apparatus of motor neurons or dorsal root ganglia have not been investigated. It is reasonable to propose that depending on the nature of the initial insult, primary axonic or presynaptic terminal lesions may eventually affect the Golgi apparatus. Alternatively, structural and/or functional lesions of the neuronal Golgi apparatus, especially those involving the processing and targeting of membrane constituents that move along the components of the fast axoplasmic transport, may affect axons and presynaptic terminals. Future studies on the above issues may be relevant to amyotrophic lateral sclerosis, other motor neuronopathies, and axonic neuropathies.

Deafferentation Experiment

Qualitative and morphometric studies have shown conclusively that the Golgi apparatus of deafferented neurons is not fragmented (Figures 7 and 8, Table 4). To the contrary, in deafferented neurons the number of immunostained Golgi elements was reduced but the ratios between surfaces occupied by the Golgi apparatus and total cell area were similar between the

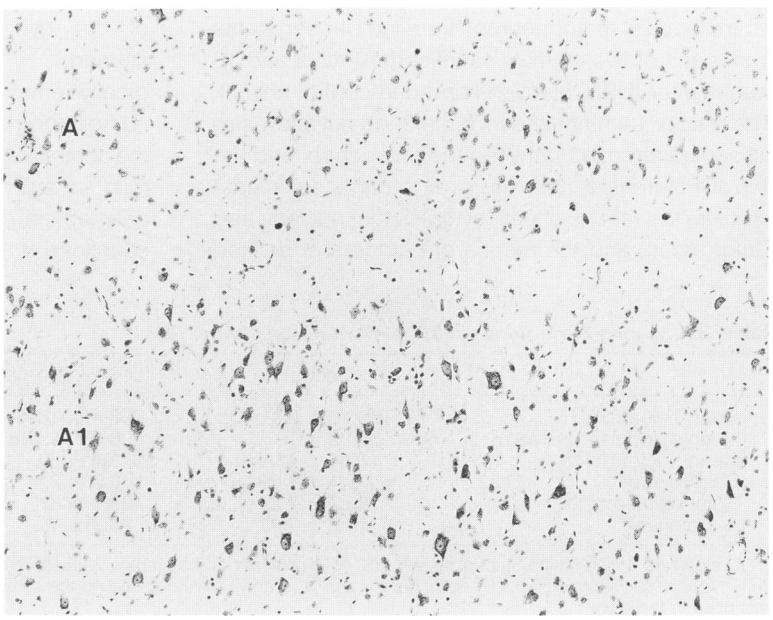

Figure 7. *Section of the left LGN of cat sacrificed 8 months after enucleation of the right eye and stained with Nissl; note atrophic neurons in layer* A *and normal neurons in adjacent layer* A1 (×400).

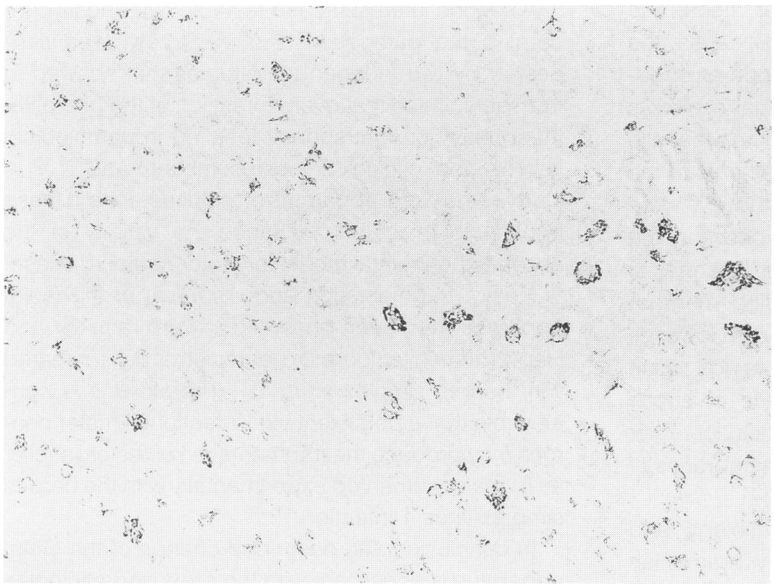

Figure 8. *Section of the left LGN of same cat as in Figure 7 immunostained for the Golgi apparatus. Atrophic neurons on the left half of the figure have smaller but not fragmented elements of the Golgi apparatus. Qualitative morphological observations were confirmed by morphometric studies (see Table 4) (×900).*

Table 4. *Morphometric Studies of the Lateral Geniculate Nuclei of Cat Sacrificed 8 Months After Enucleation*

	LLG3N, RLG3N (Normal Layers)	LLG3A, RLG3A (Atrophic Layers)	*P*
Mean cell area/μ^2	293.3 ± 118.9	112.9 ± 56.4	<0.005
Mean cytoplasmic area/μ^2	261.3 ± 106	104.1 ± 51.2	<0.005
Mean nuclear area/μ^2	31.9 ± 14.6	9 ± 5.4	<0.005
Golgi/size/μ^2	2.6 ± 1	1.7 ± 0.5	<0.005
Golgi elements/cell	16 ± 4.3	9.6 ± 3.4	<0.005
% Golgi/cell	14.8 ± 3.7	14.9 ± 3	NS

P values are based on the Student's *t*-test. NS, not significant; LLG3N, left lateral geniculate nucleus, normal layer; RLG3N, right lateral geniculate nucleus, normal layer, LLG3A, left lateral geniculate nucleus, atrophic layer; RLG3A, right lateral geniculate nucleus, atrophic layer. The 40 neurons from the normal layers and 40 from the atrophic layers were analyzed.

normal and the atrophic deafferented neurons. It should be noted that proximal axonal transection followed by chromatolysis did not result in the fragmentation of the Golgi apparatus.[6]

Clinical Studies

The clinical studies reported previously and in this paper and the experimental studies reported here strongly suggest that the dramatic fragmentation of the Golgi apparatus of motor neurons in non-Guamanian and Guamanian ALS is not due to postmortem changes, nonspecific axonic lesions, or neuronal deafferentation (Figures 1 and 2, Tables 1, 3, and 4).[8–10] Fragmentation of the organelle of motor neurons was observed with less frequency in control cases, whereas motor neurons from three cases of ALS with a prolonged clinical course did not show a similar change of the organelle (Table 1). For these reasons it is unlikely that the fragmentation of the organelle is due to malnutrition or other generalized metabolic defects associated with a chronic debilitating illness. Furthermore, this study has shown conclusively a positive correlation between the ubiquitin-containing inclusions of motor neurons thought to be pathognomonic for ALS and the fragmentation of the Golgi apparatus (Figure 3, Table 2).[9,10]

Since the original studies on the ALS-Parkinson's disease-dementia complex in Guam, numerous clinical, pathological, toxicological, and epidemiological studies have been devoted to this unusual disease.[26,27] The histopathological findings are similar in both the Guamanian and non-Guamanian ALS. Among the noteworthy changes observed in the remaining neurons include the accumulation of neurofilaments in proximal axons and perikarya and cytoplasmic inclusions consisting of Bunina bodies and ubiquitin-positive skein-like structures and/or dense bodies.[11,12] In affected motor neurons, the initial segments of axons are unusually thin and dendrites show thin processes and poor branching.[27] The nature and pathogenesis of these abnormalities is not known. However, these observations are consistent with a fragmented and presumably malfunctioning Golgi

apparatus, which is very important for membrane synthesis and axoplasmic flow.[1,2,4] Because the structural organization of the Golgi apparatus depends on microtubules and possibly other elements of the cytoskeleton, the fragmentation of the organelle may be related to changes in those elements in the neuronal perikaryon.[28,29] In an earlier study we were not able to demonstrate correlations between the fragmentation of the Golgi apparatus and changes of microtubules and neurofilaments studied morphologically with immunoenzymatic method.[9] However, negative results obtained from immunostained tissue sections do not preclude the existence of subtle changes in neurofilaments, microtubules, or other proteins involved in the attachment of membranes of the Golgi apparatus to microtubules.[29–31]

Current opinions concerning the etiology and pathogenesis of Guamanian and non-Guamanian ALS favor a combination of environmental and genetic factors. The progressive decline of the incidence of ALS among the Guam natives, from 50 to 5 per 100,000, may be attributed to the acquisition of Western customs by the Guamanians and the gradual elimination of effects of putative environmental factor(s).[32]

The high incidence of ALS in Guam has stimulated toxicological studies that have contributed to the formulation of the excitotoxic hypothesis. According to this hypothesis, chronic overstimulation of neurons by exogenous (like the excitotoxic amino acid β-*N*-methylamino-L-alanine) or endogenous (such as glutamate) ligands may gradually produce lead cell death.[32] The recent discoveries that 1) mutations in the Cu/Zn superoxide dismutase (SOD) gene are associated with certain cases of familial amyotrophic lateral sclerosis and 2) the human glutamate receptor gene, GLUR5, maps to a region of chromosome 21, which includes the locus for familial ALS, are important contributions that will generate testable experimental and therapeutic approaches.[33–36]

In an insightful analysis of recent information derived from genetic and neurotoxicological studies, McNamara and Fridovich propose that a cascade of events initiated by activation of Kainate/AMPA receptors or voltage-sensitive Ca^{2+} channels cause the accumulation of superoxide anions which are responsible for neuronal death in ALS.[37] Because superoxide ions have many biological targets, including membranes, the observed fragmentation of the Golgi apparatus of motor neurons in ALS may be due to superoxide anion toxicity caused by a chronic deficiency of superoxide dismutases.[33] This hypothesis may be tested in experimental paradigms.

A second interesting and testable hypothesis concerning the pathogenesis of ALS has been suggested by two independent studies that showed in transgenic mice that overexpression of the neurofilament heavy or light subunit resulted in pathological lesions and clinical signs resembling ALS.[39,40] A study of the Golgi apparatus of motor neurons in these transgenic mice may enhance the validity of the above experimental model for motor neuron disease.

Finally, fragmentation and dispersal of the Golgi apparatus has been observed in Vero monkey and Hep-2 human epidermoid carcinoma cells infected with herpes simplex virus 1.[41] Therefore, it is conceivable that a persistent viral infection of motor neurons may produce the fragmentation of the organelle, as it was documented experimentally with the herpes simplex virus 1 infection.[41,42]

In conclusion, the described change of the Golgi apparatus is consistent with the proposed etiologies of ALS involving mutations of the SOD gene, glutamate-induced excitotoxicity, alterations of the neuronal cytoskeleton, and persistent viral infections. We suggest that the involvement of the Golgi apparatus of spinal cord motor neurons in ALS contributes to the malfunction of the motor unit and the development of neuronal degeneration.

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