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Pathological Characterization of Astrocytic Hyaline Inclusions in Familial Amyotrophic Lateral Sclerosis

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To clarify the pathological characteristics of astrocytic hyaline inclusions (Ast-HIs) in patients with familial amyotrophic lateral sclerosis (FALS) with neuronal Lewy-body-like hyaline inclusions (LBHIs), eight autopsies on members of four different families, including two long-term surviving patients with clinical courses of over 10 years, were analyzed. Ast-HIs were found only in the two long-term surviving patients who belonged to different families and to different races. Ast-HIs were ultrastructurally composed of 15- to 25-nm granule-coated fibrils that had immunoreactivities to superoxide dismutase 1 (SOD1) and ubiquitin. Approximately 50% of the Ast-HIs expressed αB-crystallin, metallothionein, glutamine synthetase, and tubulin (α and β) at various intensities. Some Ast-HIs reacted with antibodies to tau protein, S-100 protein, and heat shock protein 27. The Ast-HIs were not stained for glial fibrillary acidic protein. Our results suggest a cooperative role of superoxide dismutase 1, ubiquitin, and cytoskeletal proteins in the formation of granule-coated fibrils (namely, Ast-HIs) and provide evidence that Ast-HIs are formed in certain long-surviving familial amyotrophic lateral sclerosis patients with neuronal Lewy-body-like hyaline inclusions. *(Am J Pathol 1997, 151:611–620)*

The Lewy-body-like hyaline inclusions (LBHIs), a characteristic neuropathological feature of familial amyotrophic lateral sclerosis (FALS) with posterior column involvement (PCI), are seen in the soma or the cord-like swollen neurites of some affected anterior horn cells.[1] LBHIs are composed of eosinophilic and/or pale areas, display round, ring-shaped, sausage-like, or ill-defined profiles,[1,2] and express epitopes of neurofilament protein (NFP) and ubiquitin.[2–5] Recent studies have demonstrated mutations in the superoxide dismutase 1 (SOD1) gene in certain cases of FALS[6] and that LBHIs are intensely immunostained by antibody to SOD1.[7–9] We reported astrocytic hyaline inclusions (Ast-HIs) resembling neuronal LBHIs in hematoxylin and eosin (H&E) preparations in a Japanese FALS patient who had an 11-year clinical course.[9] At the ultrastructural level, both neuronal LBHIs and Ast-HIs consist of 15- to 25-nm granule-coated fibrils.[9] The novel astrocytic inclusions are stained by antibodies to SOD1 and ubiquitin.[9] In addition to the Japanese patient having Ast-HIs, we have found the same Ast-HIs in an American FALS patient whose clinical course was 23 years. No detailed immunohistochemical, electron microscopic, and immunoelectron microscopic investigations are available on the Ast-HIs.

To clarify the pathological characteristics of the Ast-HIs, we have applied immunohistochemical assays to compare epitope expression by Ast-HIs and neuronal LBHIs, electron microscopic techniques to analyze the ultrastructures of Ast-HIs and neuronal LBHIs, and immunoelectron microscopic methods to determine the ultrastructural localization of SOD1 and ubiquitin in Ast-HIs. Our data suggest a cooperative role of SOD1, ubiquitin, and cytoskeletal proteins in Ast-HI formation and provide evidence that Ast-HIs are formed during the long-term disease process of certain long-surviving FALS patients with neuronal LBHIs.

Materials and Methods

Patient Material

The immunohistochemical studies were performed on archival, formalin-fixed, paraffin-embedded tissues obtained at autopsy of eight FALS patients, members of four different families. One case (case 1) was a member of the American family reported by Metcalf and Hirano (M-H family),[10] two cases (cases 2 and 3) were of the Japanese Oki family,[9,11–13] two cases (cases 4 and 5) were of

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Table 1. Characteristics of the Eight Cases of Familial Amyotrophic Lateral Sclerosis (FALS) Examined

Case	Age at death (years)	Sex	Race	Duration of FALS	SOD1 gene mutation	FALS subtype	Ast-HIs	Neuronal LBHIs	
								Motor system	Nonmotor system
1	54	F	Black	23 years	ND	PCI	+	+	+
2	65	M	Japanese	11 years	2-bp deletion* (126)	PCI and degeneration of other systems	+	+	+
3	46	F	Japanese	18 months	2-bp deletion (126)	PCI	−	+	−
4	41	F	Japanese	15 months	ND	PCI	−	−	−
5	45	F	Japanese	25 months	ND	PCI and degeneration of other systems	−	+	−
6	39	M	White	7 months	A4V†	PCI	−	+	−
7	46	M	White	8 months	A4V†	PCI	−	+	−
8	66	M	White	1 year	ND†	PCI	−	+	−

Case 1 is a member of the American M-H family, cases 2 and 3 are of Japanese Oki family, cases 4 and 5 are of the Japanese T family, and cases 6, 7, and 8 are of the American C family. F, female; M, male; ND, not determined; +, detected; −, not detected; PCI, posterior column involvement type.
*As determined previously.[9]
†As determined previously.[8]

Table 2. Antibodies Used and Their Immunoreactivity with Astrocytic Hyaline Inclusions and Neuronal Lewy-Body-Like Hyaline Inclusions

Antibody against	Clonality	Dilution	Source	Ast-HIs	Neuronal LBHIs
SOD1	P	1:10,000	Ref. 54	+	+
Ubiquitin	P	1:800	Dr. S.-H. Yen[55]	+	+
GFAP[a]				−	−
αB-Crystallin	P	1:250	Dr. J. E. Goldman[45]	+/−	−
MT	M	1:200	Dako, Glostrup, Denmark	+/−	−
GS	P	1:3,000	Dr. M. Oda[41]	+/−	−
α-Tubulin	M	1:200	Bioyeda, Rehovot, Israel	+/−	+
β-Tubulin	M	1:200	Biomakor, Rehovot, Israel	+/−	+
Tau-1	M	1:5	Dr. S.-H. Yen[56]	−/+	−/+
Tau-2	M	1:5	Dr. L. I. Binder[57]	−/+	−/+
S-100 protein	P	Ready to use	BioGenex, San Ramon, CA	−/+	−
HSP27	M	Ready to use	BioGenex	−/+	−
pNFP (SMI31)	M	1:5,000	Sternberger, Baltimore, MD	−	+
npNFP (SMI32)	M	1:500	Sternberger	−	+
Synaptophysin	M	1:100	Boehringer Mannheim, Indianapolis, IN	−	+/−
NSE	P	Ready to use	Nichirei, Tokyo, Japan	−	+/−
MBP	M	1:500	Hybritech, San Diego, CA	−	−
Leu7	M	1:200	Becton Dickinson, Mountain View, CA	−	−
CNP	P	1:3,000	Dr. T. Kurihara[58]	−	−
SOD2	P	1:10,000	Ref. 59	−	−
Actin	M	Ready to use	Enzo, New York, NY	−	−
Vimentin	M	1:200	Dako	−	−
Desmin	M	1:100	Dako	−	−
Cytokeratin (52.5 kDa)	M	1:250	Enzo	−	−
MAP1 (1A)	M	1:500	Biomakor	−	−
MAP2	M	1:1,000	Amersham, Little Chalfont, UK	−	−
MAP5 (1B)	M	1:1,000	Amersham	−	−
PHF (Ab39)	M	1:5	Dr. S.-H. Yen[60]	−	−
nNOS	P	1:250	Dr. H. Esumi[61]	−	−
iNOS	P	1:250	Dr. H. Esumi[62]	−	−
HSP60 (LK-1)	M	1:250	StressGen, Victoria, Canada	−	−
HSP60 (LK-2)	M	1:250	StressGen	−	−
HSP72	M	1:500	Amersham	−	−
HSP90	M	1:5,000	Affinity BioReagents, Neshanic Station, NJ	−	−
Chromogranin A	P	Ready to use	BioGenex	−	−
Cathepsin D	P	1:40	BioGenex	−	−
AACT	P	1:3,000	Dako	−	−
AAT	P	1:3,000	Dako	−	−
Lysozyme	P	Ready to use	Nichirei	−	−
EMA	M	Ready to use	Nichirei	−	−

Antibodies against GFAP used in this study are listed in Table 3. PHF, paired helical filament; nNOS, neuronal NOS; iNOS, inducible NOS; AACT, α1-antichymotrypsin; AAT, α1-antitrypsin; EMA, epithelial membrane antigen; P, polyclonal; M, monoclonal.
Results are expressed as follows: +, most inclusions showed strongly positive reaction; +/−, approximately 50% of inclusions showed positive reaction; −/+, some inclusions were positively stained; −, negative.

Table 3. Antibodies against Glial Fibrillary Acidic Protein (GFAP) Used

Immunogen	Clonality and Isotype	Dilution	Source
Human brain	M, IgG_1	Ready to use	Nichirei
Porcine spinal cord	M, IgG_1	Ready to use	BioGenex
Cultured human glioma cells	M, IgG_1	1:5	Milab, Malmö, Sweden
Human brain	M, IgG_1	1:5	Amersham
Human spinal cord	P, IgG	1:50	Bio-Science Products, Emmenbrüke, Switzerland
Bovine spinal cord	P, affinity-purified immunoglobulin	1:500	Dako

M; monoclonal, P; polyclonal.

the Japanese Tamatsukuri family (T family),[14] and three cases (cases 6, 7, and 8) were of the American C family.[8,15–17] The main characteristics in the eight patients are summarized in Table 1. The long-surviving patients were cases 1 and 2. Case 1 was alive when first reported,[10] and the clinicopathological findings on the other seven have been described previously (cases 2 and 3,[9,11] cases 4 and 5,[14] cases 6 and 7,[16] and case 8[17]).

Detailed clinical data of case 1 at age 27 to the age of 52 were described previously (the onset of FALS occurred at the age of 31) (case IV-31 in Ref. 10). The patient was hospitalized at age 53 because of profound muscle weakness and frequent urinary tract infection. She died 1 year later from respiratory insufficiency.

With respect to the clinical courses of cases 2 and 3 that have the same frame-shift 126 mutation,[9] case 2 was placed on a respirator due to the respiratory muscle weakness approximately 12 months after the onset and survived for 10 years with respiratory support.[9] Case 3 died of accidental asphyxiation without using a respirator, 18 months after the onset.[11]

Histopathology and Immunohistochemistry

After fixation in 10% buffered formalin, the specimens were embedded in paraffin, cut into 6-μm-thick sections, and examined by light microscopy. Brain and spinal cord sections were stained by the following routine methods: H&E, Klüver-Barrera, Holzer, phosphotungstic acid-hematoxylin, periodic acid-Schiff, alcian blue, Mallory azan, Masson trichrome, Congo red, thioflavin S, Berlin blue, Bielschowsky, and Gallyas-Braak stains. Representative paraffin sections were used for the immunohistochemical assays. The sources of the primary antibodies and the dilutions used are listed in Tables 2 and 3. Sections were deparaffinized, and endogenous peroxidase activity was quenched for 30 minutes with 0.3% H_2O_2. The sections were then washed in phosphate-buffered saline (PBS, pH 7.2). Normal sera homologous with the second antibody were used as blocking reagents. Sections were incubated with the primary antibodies for 18 hours at 4°C. Bound antibodies were visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using the appropriate Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine tetrahydrochloride as final chromogen.

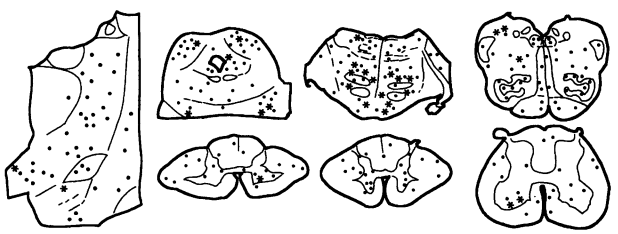

Figure 1. Schematic topographic distribution of Ast-HIs and neuronal LBHIs in the spinal cord (C5, T5, and L3), medulla oblongata, pons, midbrain, and internal capsule at the level of the subthalamic nucleus of case 1. Each dot represents an Ast-HI; neuronal LBHIs are indicated by **asterisks**.

Electron Microscopy and Immunoelectron Microscopy

For routine electron microscopy, selected areas of the brain and spinal cord were immersed at autopsy in 2.5% glutaraldehyde (in 0.1 mol/L cacodylate buffer, pH 7.4) and fixed for 12 hours at 4°C. The preparations were subsequently washed, post-fixed in 1% osmium tetroxide (in 0.1 mol/L cacodylate buffer, pH 7.4) for 2 hours at 4°C, processed by conventional methods, and embedded in epoxy resin (Epon 812). One-micron-thick sections were stained with toluidine blue and examined by light microscopy. Selected areas were identified and trimmed, and ultrathin sections were prepared with a diamond knife using a Sorvall MT2-B ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-300 electron microscope.

tk;2For immunoelectron microscopy by the indirect immunoperoxidase decoration method, paraffin sections on glass slides were stained with the antibodies against SOD1 and ubiquitin using diaminobenzidine as the chromogen, post-fixed for 2 hours with 2% osmium tetroxide, processed by conventional methods, and embedded in Epon. For indirect immunogold labeling, the paraffin sections were stained with the antibody to SOD1 and incubated with biotinylated goat anti-rabbit IgG (1:100; Vector) for 72 hours at 4°C and then for 60 minutes at room temperature with streptavidin conjugated to 15-nm colloid gold particles (1:5; British-BioCell, Cardiff, UK). The samples were rinsed, dehydrated in ethanol, and embedded in Epon. Embedding was done by inverting gelatin capsules filled with Epon onto the target areas of the sections; polymerization was carried out at 50°C for 24 hours, followed by hardening at 60°C for another 24 hours. The Epon-embedded areas were detached from

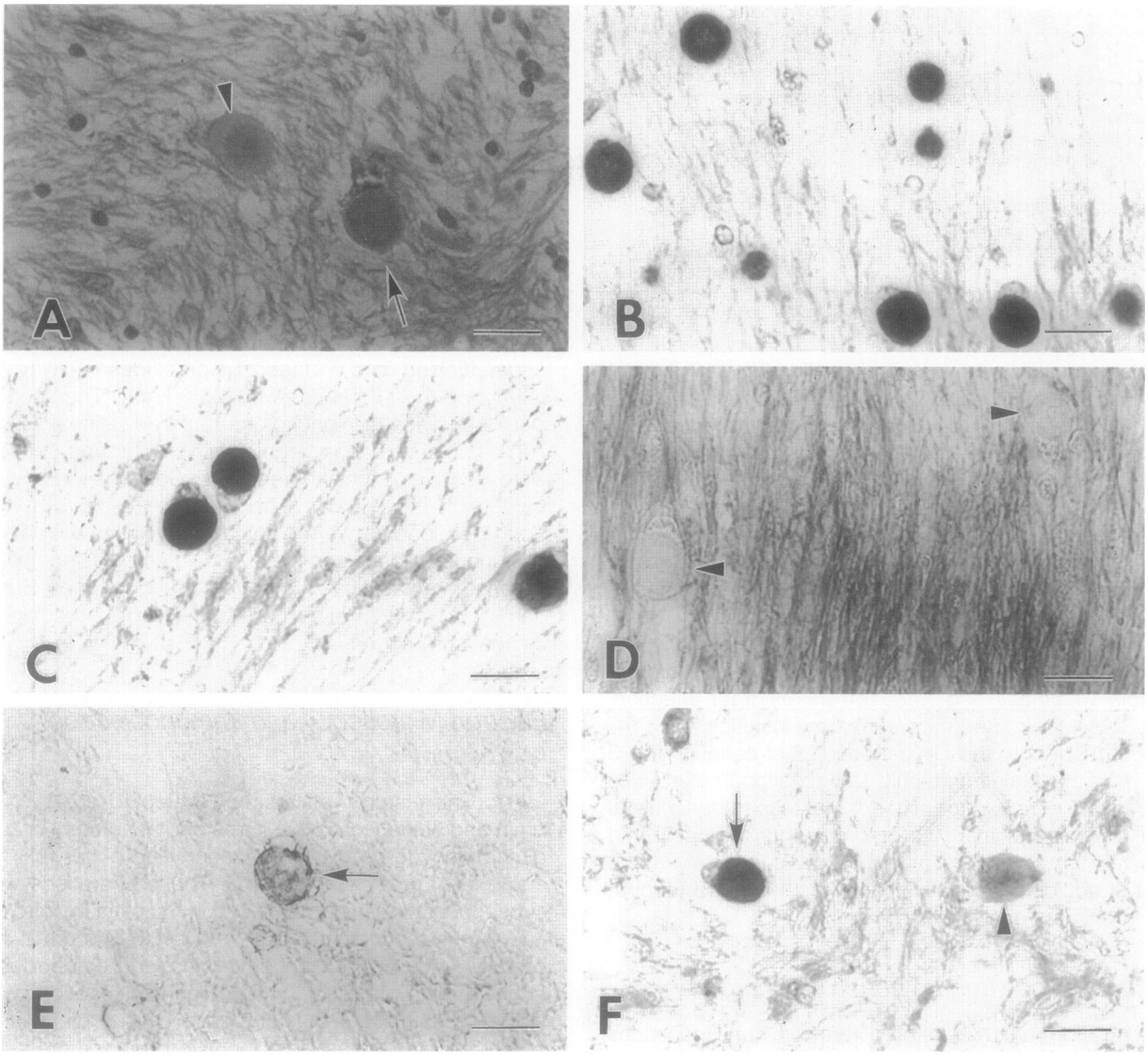

Figure 2. Light microscopic characteristics of Ast-HIs. **A:** Typical Ast-HIs in H&E preparations. One Ast-HI is round and eosinophilic (**arrow**), and the other has an eosinophilic core with a paler peripheral halo (**arrowhead**). **B:** Immunostaining with the antibody against SOD1. The inclusions are intensely stained by the antibody. **C:** Immunostaining with the antibody to ubiquitin. The Ast-HIs are intensely positive. **D** and **E:** Immunostaining with the antibody to GFAP. The inclusions themselves are not stained (**arrowheads** in **D**), but the periphery is stained (**arrow** in **E**). **F:** Immunostaining with the antibody against αB-crystallin. One Ast-HI is intensely stained (**arrow**), and another one only weakly stained (**arrowhead**). **G:** Ast-HIs immunostained by the antibody to MT; one is strongly positive (**arrow**), and the other is weakly positive (**arrowhead**). **H:** Ast-HIs immunostained by the antibody against GS. One of them expresses GS strongly (**arrow**), and the other weakly (**arrowhead**). **I:** Immunostaining with the antibody to α-tubulin. One Ast-HI is intensely stained (**arrow**) and another one weakly (**arrowhead**). **J:** Immunostaining with the antibody against tau protein (tau-1). There is a clearly stained Ast-HI (**arrow**) and two inclusions that are very faintly stained or not at all (**arrowheads**). **K:** Immunostaining with the antibody to S-100 protein. One inclusion is positive (**arrow**), and the others are negative (**arrowheads**). **L:** Immunostaining with the antibody against HSP 27. A clearly positive inclusion (**arrow**) and very weakly positive or negative inclusions (**arrowheads**) can be seen. Bar, 15 μm (**A** to **L**).

the glass slides by heating with a gas burner. The presence of immunoreactive structures was confirmed by light microscopy. The Epon blocks were trimmed, cut into ultrathin sections, and examined with an electron microscope.

SOD1 Gene Analysis

Genomic DNA of FALS cases 1, 4, and 5 was extracted from formalin-fixed, paraffin-embedded tissues.[18] The primer sequences for polymerase chain reaction (PCR) amplification of exons 1 and 5 of SOD1 are given in Ref. 19, those of exons 2 and 4 in Ref. 20, and those of exon 3 in Ref. 21. The PCR products were electrophoresed on 20% polyacrylamide gels containing 5% glycerol for 20 to 24 hours at 4°C and 6 W. The DNA bands were detected by the standard silver staining method.

Results

Neuropathological Observations

Although the brain weight of case 1 was not available, the brain had a normal appearance externally. The spinal

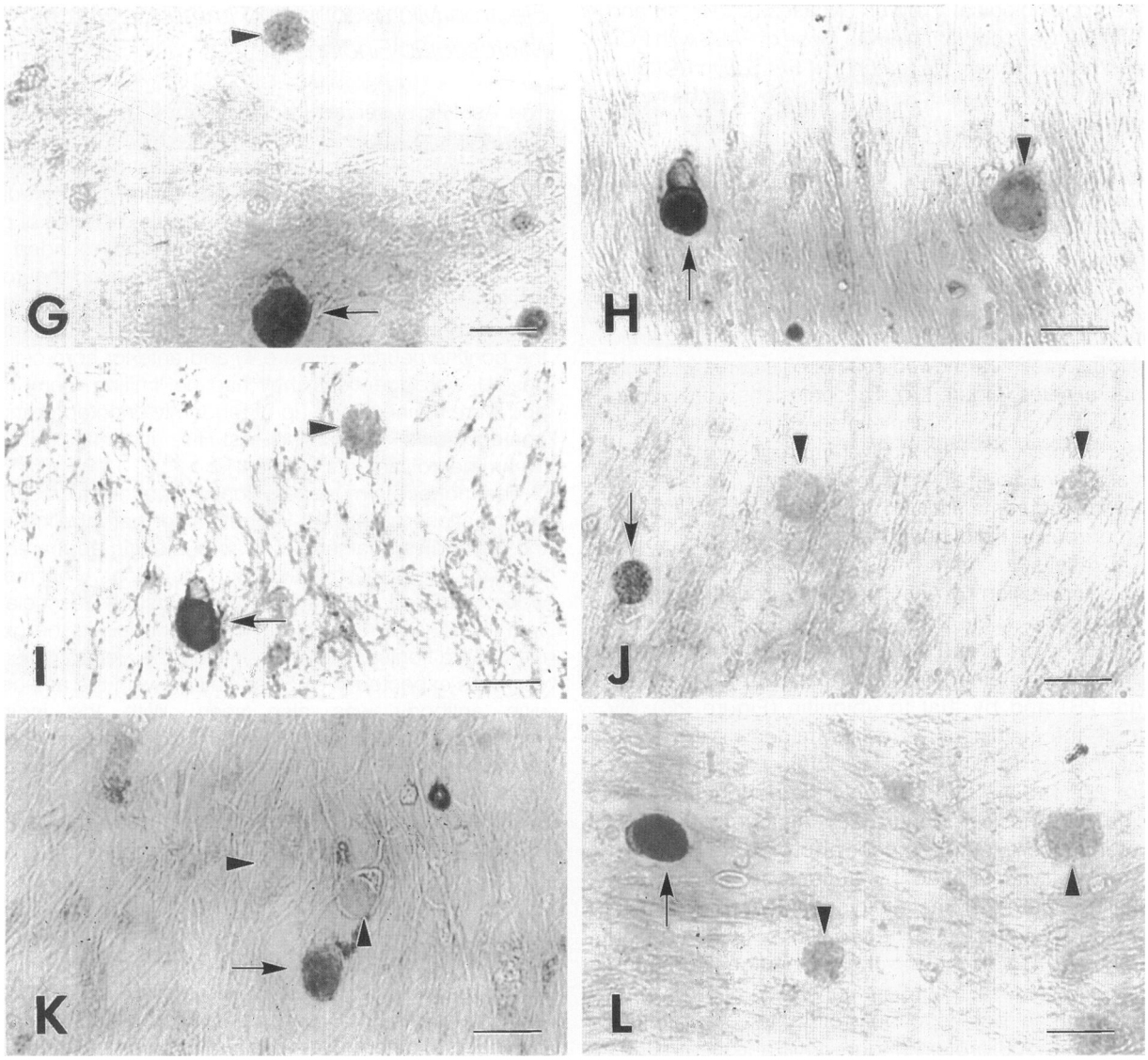

Figure 2. Continued

cord appeared atrophic, and there was wasting of the anterior roots. On coronal sections, no apparent abnormalities were found in the cerebrum, brain stem, and cerebellum.

The obvious histopathological changes of case 1 were evident in the spinal cord and revealed features of FALS with PCI. They included a decreased number of lower motor neurons in the spinal cord and brain stem, loss of neurons in Clarke's nuclei, and degeneration of the pyramidal tracts, posterior spinocerebellar tracts, and posterior columns, especially in the so-called middle root zone in the lower thoracic to lumbosacral segments (Figure 1). There was bilateral degeneration of Goll's tracts. Although most remaining lower motor neurons were shrunken and filled with lipofuscin, some of them contained LBHIs. Additionally, LBHIs were also seen in many pontine neurons and in a few neurons in the tegmentum of the brain stem (see Figures 1 and 4). The most striking finding of case 1 was that there were many Ast-HIs observed beyond the motor neuron system (Figure 1). The thoracic intermediolateral and sacral autonomic nuclei of the spinal cord appeared to be well preserved. The posterior horns showed no abnormalities. The anterior roots showed moderate loss of myelinated fibers. The oculomotor nuclei were intact. Other parts of the brain were unremarkable. Muscular tissues were not available.

No PCR-mediated amplification of any of the SOD1 exons tested was obtained with the genomic DNA extracted from the formalin-fixed, paraffin-embedded tissues of cases 1, 4, and 5 (Table 1).

Of the eight FALS cases examined, Ast-HIs were seen only in cases 1 and 2. The inclusions were evident in preparations stained with H&E as well as in those immunostained with the antibodies against SOD1 and ubiquitin. By comparison, neuronal LBHIs were observed in seven cases, and none were seen in case 4 (Table 1).

The neuropathological features of cases 3,[11] 4,[14] 6 and 7,[16] and 8[17] were compatible with those of FALS with PCI. Cases 2[9] and 5[14] had the features of FALS with PCI plus degeneration of other neuron systems beyond the motor neuron system (Table 1).

Histopathological and Immunohistochemical Findings

The Ast-HIs of cases 1 and 2 were identical. They were eosinophilic or slightly paler inclusions and when stained with H&E sometimes showed eosinophilic cores with pale peripheral halos (Figure 2A). The inclusions were generally round to oval and sometimes sausage-like. Most Ast-HIs were argyrophilic in silver-impregnated preparations and were generally blue to violet after Mallory azan or Masson trichrome staining. They were not stained by the other routine histochemical methods.

The results of immunohistochemical assays on epitope expression by Ast-HIs and neuronal LBHIs are summarized in Table 2. The immunoreactivities of the Ast-HIs of cases 1 and 2 were identical. Most Ast-HIs were intensely stained by the antibody against SOD1 (Figure 2B) and by that to ubiquitin (Figure 2C). By contrast, the inclusions were not stained by six different antibodies to glial fibrillary acidic protein (GFAP; Figure 2D; Tables 2 and 3); only the periphery of few Ast-HIs were positive (Figure 2E). Although the intensity of immunoreactivity varied, approximately 50% of the Ast-HIs was labeled by antibodies against αB-crystallin (Figure 2F), metallothionein (MT; Figure 2G), glutamine synthetase (GS; Figure 2H), or tubulin (α and β; Figure 2I). Even though the proportion of stained inclusions was less, approximately 10 to 30% of them reacted with antibodies to tau protein (tau-1 and tau-2; Figure 2J), S-100 protein (Figure 2K), and heat-shock protein 27 (HSP 27; Figure 2L). The Ast-HIs did not react with antibodies against phosphorylated NFP (pNFP), nonphosphorylated NFP (npNFP), synaptophysin, neuron-specific enolase (NSE), myelin basic protein (MBP), Leu 7, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), mitochondrial Mn-containing superoxide dismutase 2 (SOD2), actin, vimentin, desmin, cytokeratin (52.5 kd), microtubule-associated protein 1 (MAP-1 (1A)), MAP-2, MAP-5 (1B), paired helical filament, neuronal nitric oxide synthase (NOS), inducible NOS, HSP 60 (LK-1 and LK-2), HSP 72, HSP 90, chromogranin A, cathepsin D, α1-antichymotrypsin, α1-antitrypsin, lysozyme, and epithelial membrane antigen.

By contrast and corroborating recent findings,[2,4,5,7–9] the neuronal LBHIs were immunostained for SOD1, ubiquitin, and pNFP as well as npNFP. In addition, most LBHIs were also stained by antibodies to α- and β-tubulin; approximately 50% were positive for synaptophysin and NSE. Although the proportion of stained inclusions was less, a positive reaction was observed with the antibody against tau protein (tau-1 and tau-2).

Electron Microscopic and Immunoelectron Microscopic Findings

The Ast-HIs observed in cases 1 and 2 had the same ultrastructure. They were composed mainly of 15- to 25-nm granule-coated fibrils. In most astrocytes bearing the inclusions, the cytoplasm was entirely replaced by the granule-coated fibrils. In few Ast-HIs, bundles of glial fibrils as normal intermediate filaments surrounded the inclusions (Figure 3; Table 4). By comparison, neuronal LBHIs were found not only in anterior horn cells of cases 1 and 2 but also in nonmotor neurons. The LBHIs seen in the pontine neurons (Figure 4) and anterior horn cells of case 1 were identical; they had no limiting membrane and were mainly made up of randomly oriented granule-coated fibrils resembling Ast-HIs, intermingled with 10-nm neurofilaments (Table 4).

The indirect immunoperoxidase electron microscopic examinations of the Ast-HIs revealed that labeling with the antibodies against SOD1 and ubiquitin appeared as an osmiophilic meshwork of the inclusions. The meshwork consisted of coarse linear structures associated with ill-defined granules that corresponded to the granule-coated fibrils observed using routine electron microscopy. As expected,[22] nuclear staining with the anti-ubiquitin antibody was also seen. With the indirect immunogold technique, colloidal gold particles labeled by the antibody to SOD1 were found on the surface of the granule-associated thick fibrils that corresponded to the granule-coated fibrils observed using routine electron microscopy (Figure 5).

Discussion

As our results indicate, Ast-HIs were found in cases 1 and 2 of the eight FALS patients studied. The two cases belonged to different families and to different races. Two common characteristics distinguish cases 1 and 2 from the other six FALS cases. One is that both were long-surviving patients with clinical courses of over 10 years, and the other is that they had neuronal LBHIs in the nonmotor systems as well as in the motor neuron system. The two characteristics would imply that the relatively long duration of the illness in certain FALS patients who have neuronal LBHIs causes LBHI formation to extend beyond the motor neuron system. Confirming the notion that the degeneration process of FALS extends to astrocytes,[9] formation of the abnormal granule-coated fibrils that are the main constituents of the neuronal LBHIs extends to astrocytes in two unrelated long-surviving FALS patients. A similar situation also occurs in Alzheimer's disease. Thus, paired helical filaments, major components of neurofibrillary tangles that develop exclusively in neurons, were observed in astrocytes of a long-surviving Alzheimer disease patient with the clinical course of 25 years.[23] Similarly, straight tubules indistinguishable from those of neurofibrillary tangles in progressive supranuclear palsy (PSP) were noted in astrocytes even of PSP patients.[24] Although we cannot readily compare

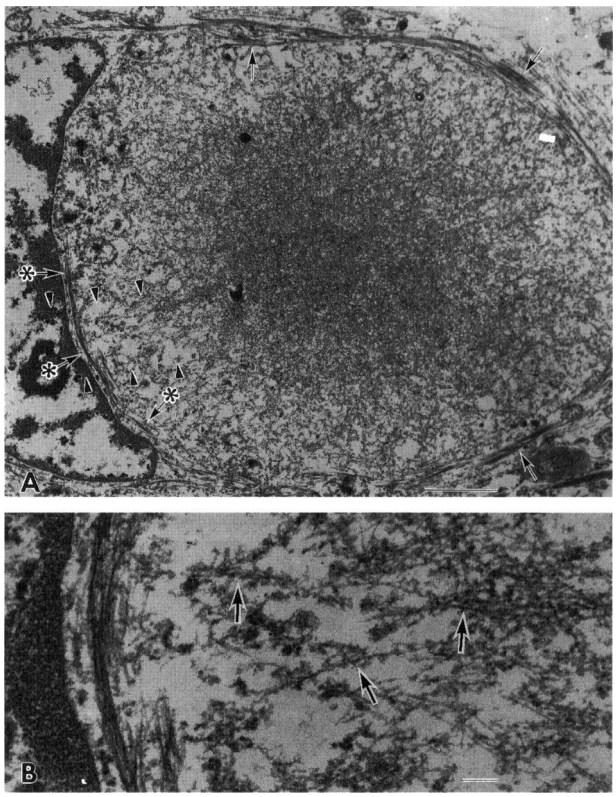

Figure 3. Electron micrograph of an Ast-HI of case 1. The inclusion components are densely aggregated in the central portion, probably corresponding to the core. **A**: The major components of the Ast-HI are fibrils and granular materials. Bundles of glial fibrils surround the Ast-HI (**arrows**), and some small bundles of glial fibrils are present between the inclusion and the nucleus (**arrows** and **asterisks**). This electron microscopic finding represents the immunohistochemical observation that only the peripheral structure of the Ast-HI is positive for GFAP in Figure 2E. Bar, 2.5 μm. **B**: Higher magnification of the portion indicated by **arrowheads** in **A**. The fibrils appear composed of approximately 15- to 25-nm granule-coated fibrils (**arrows**). Bar, 200 nm.

FALS with diseases such as Alzheimer's disease and PSP, it would be suggested that processes similar to those that attack neurons also affect astrocytes, ultimately leading to the formation of granule-coated fibrils that appear as Ast-HIs.

There are transgenic mice[25–27] that express mutant human SOD1, and both the high expressor transgenic mice and the lower expressor mice have abnormal hyaline inclusion bodies in neurons at the end stage of the disease.[26,27] Mice overexpressing the wild type, however, did not have any inclusions.[26,27] These experiments would suggest that mutations in the SOD1 gene are related to the formation of the abnormal hyaline inclusions. Ast-HI formation in FALS patients with a SOD1 gene mutation could be due to the reactive free radicals elicited by reduced enzyme activity of mutant SOD1[20,28] and/or mutant SOD1 with a toxic function.[19,25,29–33] In our cases, a mutation in the SOD1 gene was previously identified in cases 2 and 3.[9] With the same methods applied to the tissue aliquots of cases 1, 4, and 5, no PCR-mediated DNA amplification was obtained. This is probably due to breakdown of genomic DNA by prolonged fixation. Nevertheless, it is likely that case 1 might have had the SOD1 gene mutation, because many LBHIs were observed in neurons in motor and nonmotor systems. Verification of this will require additional studies.

Table 4. Ultrastructural Components of Astrocytic Hyaline Inclusions and Neuronal Lewy-Body-Like Hyaline Inclusions

	Ast-HIs	Neuronal LBHIs
Abnormal filaments	Granule-coated fibrils (15 to 25 nm in diameter)	Granule-coated fibrils (15 to 25 nm in diameter)
Intermediate filaments	Glial fibrils*	Neurofilaments†

*Bundles of glial fibrils rarely surround the Ast-HIs.
†The neurofilaments are always intermixed.

The present study reveals that almost all Ast-HIs are labeled by the antibodies against SOD1 and ubiquitin and that the granule-coated fibrils, the major constituents of the Ast-HIs, had the epitopes of SOD1 and ubiquitin, as seen immunoelectron microscopically. This might mean that SOD1 and ubiquitin are integrated into the granule-coated fibrils as core proteins. As for their colocalization, ubiquitin, inducible in oxidative stress[34] that participates in the ATP-dependent proteolytic system responsible for the degradation of abnormal cell proteins,[35,36] would have a role in SOD1 degradation. Therefore, both proteins could interact with each other and be involved in the formation of the granule-coated fibrils.

We found differences between Ast-HIs and neuronal LBHIs with respect to epitope expression. Thus, whereas Ast-HIs were positive for MT, GS, S-100 protein, αB-crystallin, and HSP 27, neuronal LBHIs were not. MT, an approximately 6-kd protein containing 6 to 8% metals,[37] has been identified in liver, small intestine, and kidney.[38] This protein is rich in cysteine residues, binding and detoxifying metals such as copper, zinc, cadmium, and mercury.[39] It has been reported that MT is readily detectable in a subgroup of astrocytes of the normal human brain.[40] GS is present in the astrocytes[41] and participates in the metabolism of the neurotransmitters, glutamate, and glutamate-aminobutyric acid, as well as in ammonia detoxification.[42] S-100 protein is an acidic calcium-binding protein that is present in the nervous tissues of a wide variety of animals,[43] especially as a constituent of astrocytes.[44] There is evidence that αB-crystallin is a major component of Rosenthal fibers of astrocytes[45] and that it is present in astrocytic elements of brain tumors[46–48] and in reactive astrocytes.[49] HSP 27, which bears some amino acid sequence homology with αB-crystallin, is present in astrocytic tumor cells.[47,48,50] Taken together, our positive immunohistochemical findings on Ast-HIs indicate that these inclusions express several astrocytic markers. This suggests that the granule-coated fibrils forming the Ast-HIs contain the astrocyte-associated proteins, in addition to the finding that SOD1 and ubiquitin are present as core proteins of the fibrils. However, it is of interest that the granule-coated fibrils themselves were not stained for GFAP, as none of six anti-GFAP antibodies gave a positive reaction except with the inclusions' periphery. This reflects the ultrastructural observation that glial bundles were seen surrounding the granule-coated fibrils.

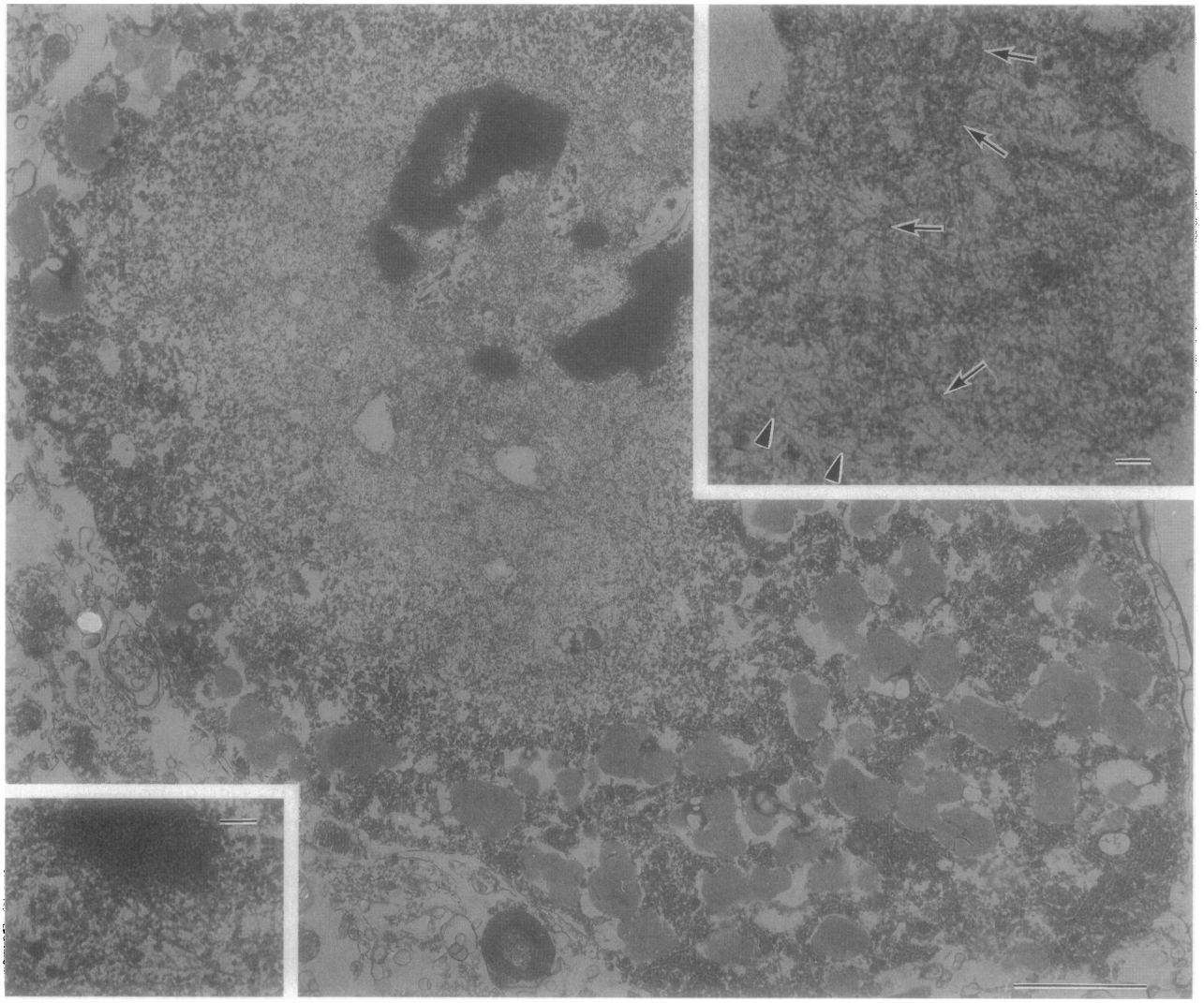

Figure 4. Electron micrograph of a LBHI in a pontine neuron of case 1. The inclusion body has a globular structure without a limiting membrane and consists mainly of fibrils and granular materials. Bar, 3 μm. The major components of the LBHI are 15- to 25-nm granule-coated fibrils (**top right inset, arrows**) and 10-nm neurofilaments (**top right inset, arrowheads**). Bar, 200 nm. An electron-dense material permeated by the granule-coated fibrils, corresponding to a part of the core, can be seen (**bottom left inset**). Bar, 200 nm. Each of the **top right** and **bottom left insets** shows the higher magnification areas in the LBHI.

Ast-HIs did not express epitopes of oligodendroglial markers such as MBP, Leu 7, and CNP. In addition, the Ast-HIs did not express neuronal markers, including pNFP, npNFP, synaptophysin, and NSE, which are present in neuronal LBHIs. On the other hand, both Ast-HIs and neuronal LBHIs expressed tubulin (α and β) and tau protein (tau-1 and tau-2). Tubulin is a constitutive cytosolic protein of neurons and astrocytes that diffuses passively into Ast-HIs and neuronal LBHIs and is not specifically deposited or otherwise sequestered in granule-coated fibrils. Similar possibilities may be involved in the expression of tau protein, one of the low-molecular-weight microtubule-associated proteins. In addition, tau-positive astrocytes have been reported to be present throughout the brain of patients with Alzheimer's disease[51,52] and PSP.[53]

The essential common constituents between Ast-HIs and neuronal LBHIs were granule-coated fibrils. Although neuronal LBHIs were always intermixed with neurofilaments, the cytoplasm of inclusion-bearing astrocytes were almost entirely replaced by granule-coated fibrils or, in few Ast-HIs, glial fibrils surrounded the inclusions. The comparative immunohistochemical studies of Ast-HIs and neuronal LBHIs suggest that, when granule-coated fibrils are formed as Ast-HIs in astrocytes, the inclusions express several astrocytic markers, and when they are produced as neuronal LBHIs in neurons, the neuronal inclusions express certain neuronal epitopes. Therefore, the criteria for the astrocytic identity of the cells having granule-coated fibrils are as follows: 1) the inclusions express certain astrocyte-related proteins but neither neuron- nor oligodendroglia-associated proteins, and 2) on rare occasions, GFAP-positive structures at the immunohistochemical level or glial fibrils at the ultrastructural level surround the inclusions. Unlike in neurons, however, the formation of granule-coated fibrils in astrocytes requires a long period in certain FALS patients. Although additional pathological and molecular analyses

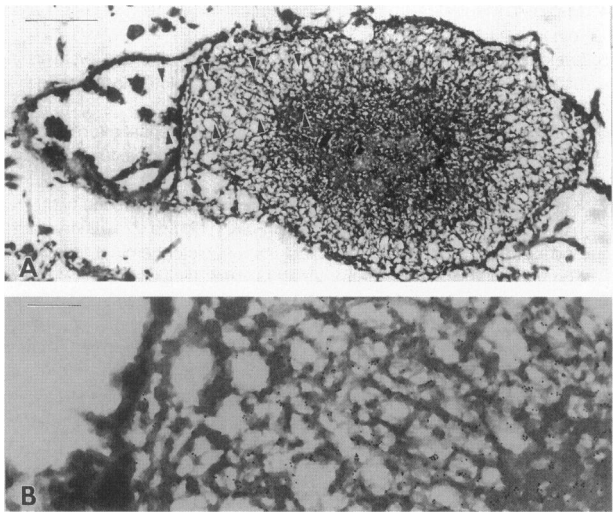

Figure 5. Immunoelectron micrograph of indirect immunogold labeling of an Ast-HI by the antibody against SOD1. **A:** Low-power magnification of an Ast-HI. Bar, 3 μm. **B:** At higher magnification (portion indicated by **arrowheads** in **A**), colloidal gold particles are present only on the surface of the granule-associated thick fibrils that correspond to the granule-coated fibrils shown in Figure 3. Bar, 500 nm.

of a large number of FALS patients are necessary to elucidate the ultimate significance of the present results, our data indicate that SOD1 is a component of granule-coated fibrils (namely, Ast-HIs) and suggest that this protein and ubiquitin as well as certain astrocyte-related and constitutive cytoskeletal proteins are involved in the inclusions' formation. Moreover, our results also furnish documentation that Ast-HIs are formed in certain long-surviving FALS patients who have neuronal LBHIs.

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