INTRODUCTION

Accumulations of hyperphosphorylated tau protein in neurons or glial cells are the hallmark lesions of a subset of neurodegenerative disorders that include corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), Pick disease, Alzheimer disease (AD), argyrophilic grain disease (AGD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (1), and parkinsonism–dementia complex of Guam (PDC). These are referred to collectively as tauopathies. Biochemical and immunohistochemical analyses of tauopathy brains have shown that the morphologically distinct inclusions consist of either all 6 brain tau isoforms or the 3R tau or 4R tau isoforms only, depending on the disease (2).

Severe cerebral cortical atrophy is observed in the gray matter of PDC brains, as well as neuronal loss predominantly in the temporal and frontal lobes, and numerous neurofibrillary tangles (NFTs) with a distribution similar to that observed in AD brains (3, 4). The distribution of cortical NFTs predominantly in layers II and III in PDC brains is similar to that seen in PSP (5). The presence of granular hazy astrocytes has been reported by Oyanagi et al to be a specific neuropathologic marker of PDC (6). Thus far, no other specific marker for PDC has been found.

In contrast to the small number of tau-positive glial inclusions observed in the AD white matter, the PDC brains contained a substantial population of tau-positive glial inclusions such as argyrophilic threads, coiled bodies, and granular hazy astrocytes. During a precise examination of the PDC white matter, we observed tau-positive fine granules (TFGs). We investigated whether these structures were universal in all patients with PDC and examined whether they are specific to this particular tauopathy. Moreover,
a biochemical analysis of tau proteins in the white matter from patients with PDC was carried out to elucidate whether these TFGs are biochemically different from other tau-positive constituents.

**MATERIALS AND METHODS**

**Cases**

The present study was carried out using brains taken at autopsy from patients with PDC (n = 35), Guamanian PDC with amyotrophic lateral sclerosis (ALS; n = 4), Guamanian ALS (n = 7), Guamanian non-PDC, non-ALS controls with neurologic disorders (n = 11), CBD (n = 10), PSP (n = 15), Pick disease (n = 4), AD (n = 10), AGD (n = 5), and myotonic dystrophy (n = 5; Table). Myotonic dystrophy was known to have NFTs in the neocortex and in subcortical nuclei (7). All of the Guamanian cases were examined and their condition diagnosed clinicopathologically by the authors (8–13). Some of the clinical and neuropathologic findings of the cases of CBD, PSP, Pick disease, AD, AGD, and myotonic dystrophy have already been reported elsewhere (14–26).

**Histochemistry and Immunohistochemistry**

The frontal white matter from autopsy brain tissue was cut into blocks, fixed in formalin, embedded in paraffin, and then sectioned at 4 µm. Some of these sections were stained with hematoxylin and eosin and by the Klüver-Barrera and Gallyas-Braak methods. The remaining sections were incubated with one of the following primary antibodies: anti-tau (AT8, monoclonal, 1:1000; Innogenetics, Temse, Belgium), anti-human tau (a gift from Professor Ihara, 1:1000; [27]), anti-ubiquitin (polyclonal, rabbit, 1:1000; Dako). The immunolabeled sections were observed with the aid of a fluorescence microscope coupled with rhodamine [1:200; Cappel, Irvine, CA] and anti-rabbit IgG coupled with rhodamine [1:200; Cappel]).

**Quantitative Examination of Neurofibrillary Tangles and Tau-Positive Fine Granules**

We performed quantitative analyses on the frontal subcortical white matter of brains from clinically diagnosed patients with PDC. The relationship between NFTs and TFGs was clarified by calculating the density of NFTs and TFGs in the frontal cortex of 12 patients with PDC. These 12 patients were sampled randomly from the 35 PDC cases examined in this study. With the aid of Gallyas-Braak staining, we computed the density of NFTs, including pretangles, in all layers of the 100-µm-wide frontal cortical ribbon. The number of TFGs in the center of the centrum semiovale in the frontal white matter was calculated by summing the number of TFGs in evenly distributed serial fields measuring 2.5 µm × 2.5 µm (giving a total area of 6.25 µm²). The correlation between the density of NFTs and that of TFGs was estimated using Spearman’s rank correlation coefficient. We used the Kruskal-Wallis test for comparing the density of NFTs with that of TFGs. Differences at p < 0.05 were considered significant.

**Immunoelectron Microscopy**

Paraffin-embedded, 6-µm-thick sections from the cerebral frontal white matter of PDC cases with tau-positive TFGs were immunostained with anti-tau antibody (AT8). The immunolabeling was visualized with diaminobenzidine (DAB), like for light microscope immunohistochemistry, and then processed for immunoelectron microscopy. After being post-fixed in 4% OsO₄ for 15 minutes, the sections were dehydrated in a graded ethanol series, embedded in epon 812, and then polymerized at 60°C for 24 hours. Ultrathin sections were cut and then stained with 3% lead acetate for 2 minutes and viewed with an electron microscope (H-9000; Hitachi, Japan) (28).

**Biochemical Analysis**

Frozen brain tissues from the frontal region, including both the gray matter and deep white matter of 4 PDC cases, one Guamanian ALS case with abundant TFGs in both the gray and white matter, one Guamanian control case, and 2 classic AD cases, were used for biochemical analysis. All of these brains were frozen at autopsy at −80°C. The gray and white matters were separated from each other macroscopically. Sarkosyl-insoluble tau was prepared according to a modification of the method of Goedert et al (29). Tissues were homogenized in a 10-fold (v/w) dilution of extraction buffer (10 mM Tris-HCl [pH 7.5], 1 mM EGTA, 0.8 M NaCl, 10% sucrose) and centrifuged at 23,000 × g for 20 minutes at 4°C. The pellets were rehomogenized in extraction buffer. Both of the 23,000 × g supernatants were combined, brought to 1% sarkosyl, and incubated for 1 hour at room temperature. After centrifugation at 113,000 × g for 20 minutes at 25°C, the

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**TABLE. Summary of Cases Examined in This Study**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number</th>
<th>Gender of Cases</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDC of Guam</td>
<td>35</td>
<td>23 male/2 female</td>
<td>64.4 ± 8.32</td>
</tr>
<tr>
<td>(died 1979–1982)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guam PDC-ALS</td>
<td>4</td>
<td>2 male/2 female</td>
<td>63.0 ± 6.06</td>
</tr>
<tr>
<td>Guam ALS</td>
<td>7</td>
<td>3 male/4 female</td>
<td>52.1 ± 10.0</td>
</tr>
<tr>
<td>Guam control</td>
<td>11</td>
<td>4 male/7 female</td>
<td>68.8 ± 11.5</td>
</tr>
<tr>
<td>Corticobasal degeneration</td>
<td>10</td>
<td>4 male/6 female</td>
<td>65.7 ± 5.43</td>
</tr>
<tr>
<td>Progressive supranuclear palay</td>
<td>15</td>
<td>8 male/7 female</td>
<td>74.4 ± 8.82</td>
</tr>
<tr>
<td>Pick disease</td>
<td>4</td>
<td>2 male/2 female</td>
<td>71.5 ± 3.70</td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>10</td>
<td>1 male/9 female</td>
<td>74.6 ± 15.1</td>
</tr>
<tr>
<td>Argyrophilic grain disease</td>
<td>5</td>
<td>3 male/2 female</td>
<td>82.4 ± 7.92</td>
</tr>
<tr>
<td>Myotonic dystrophy</td>
<td>5</td>
<td>3 male/2 female</td>
<td>54.4 ± 19.7</td>
</tr>
</tbody>
</table>

PDC, Parkinsonism-dementia complex; ALS, amyotrophic lateral sclerosis.
pellets were resuspended in 7 M guanidine-HCl and then
dialyzed overnight against 30 mM Tris-HCl (pH 8.8). De-
phosphorylation and immunoblotting were performed as de-
scribed previously (30).

RESULTS

Microscope Study

The cerebral white matter of most patients with PDC exhibited no evident pallor with Klüver-Barrera stain. In addition to the TFGs, in the white matter of PDC (Fig. 1A, C), we found some tau-positive argyrophilic threads and coiled bodies. Some tiny tau-positive granular or thread-like structures with a diameter of 1 to 3 μm were also found in the PDC brains and in those of the other tauopathies studied here (Fig. 1B, D). These thread-like structures were found mainly in the frontal and temporal white matter and were not specific to PDC. They were also observed in the white matter of brains from patients with the other tauopathies examined in this study (AGD and CBD brain) and were clearly distinct from TFGs (Fig. 2A, B).

We observed TFGs in the frontal white matter of 30 of 35 patients with PDC (86%) and in 3 of 4 patients with PDC-ALS (75%). Moreover, only one of 7 Guamanian patients with ALS (14%) and 2 of 11 Guamanian controls (18%) whose cerebral cortices exhibited many NFTs also exhibited TFGs (Fig. 3). However, no TFGs were found in the brains of patients with myotonic dystrophy, Pick disease, or AD. In CBD brains, we found large numbers of argyrophilic threads and coiled bodies, but no TFGs (Fig. 2B). The TFGs were

globe-shaped and approximately 3 to 6 μm in diameter, and were distinct from argyrophilic threads. TFGs were positive to both AT8 and anti-human tau antibodies (Fig. 1A, B), but some parts of them were not stained by the Gallyas-Braak method. Some TFGs consisted of globular dense tau-positive structures surrounded by weakly tau-positive fluffy materials (Fig. 1C). They were observed frequently in the frontal white matter, the frontal lobe, and the temporal subcortical white matter. In the PDC cases in which TFGs were abundant in the frontal white matter, some were also found in the frontal cortex. However, TFGs were only rarely observed in the spinal cord, cerebellum, or brainstem.

Confocal scanning microscope observations of immunofluorescence double-labeled sections showed that GFAP and phosphorylated neurofilament were not localized on TFGs (Fig. 4A, B). Although most of the TFGs exhibited no colocalization of ubiquitin and tau, it was observed on some (Fig. 4C). The pattern of this colocalization, when it was observed, varied from only a small part (i.e. the center) of the TFG to staining in almost all of it.

Quantitative Analysis of Tau-Positive Fine Granules and Neurofibrillary Tangles

We found no significant relationship between the number of NFTs and the number of TFGs. However, the 12 patients with PDC could be divided into 2 groups: those with more than 200 NFTs/100 μm-length of the frontal cortical ribbon and those with less (Fig. 5). The graph shown in Figure 4 shows that TFGs were only observed in those PDC cases with more than approximately 200 NFTs/100 μm-length of the frontal cortical ribbon. There was no significant
-correlation between the degree of white matter degeneration and the density of TFGs.

**Immunoelectron Microscope Observations**

Most of the AT8-positive TFGs had some contact with the myelin outer loop, but no TFGs were observed within the myelin sheath or the axons (Fig. 6A). High-power views of these sections revealed that TFGs contained round structures that were 20 to 30 nm in diameter (including the DAB substrate) (Fig. 6B). These structures were also observed near the nucleus of glial cells that were thought to be oligodendroglia (Fig. 6C, D).

**Biochemical Analysis**

The sarkosyl-insoluble fraction prepared from the white matter of the frontal lobes of PDC cases that exhibited TFGs were analyzed by Western blotting with a phosphorylation-independent anti-tau antibody HT-7. Two major bands were detected, one with an apparent molecular mass of 60 kDa and another of 64 kDa, and one minor band with an apparent molecular mass of 68 kDa. After dephosphorylation, these bands appeared as one major band corresponding to a 4-repeat tau isoform with zero amino acid inserts (4R0N) and 3 minor bands corresponding to a 4-repeat tau isoform with 29 amino acid inserts (4R29N), a 3-repeat tau isoform with zero amino acid inserts (3R0N), and a 3-repeat tau isoform with 29 amino acid inserts (3R29N; Fig. 7).

The insoluble tau extracted from the gray matter of cortices from the PDC cases resolved into 3 bands of apparent molecular mass 60, 64, and 68 kDa. Six bands were detected after dephosphorylation, corresponding to 6 tau isoforms that resembled those that were resolved in AD brains. Similar results were obtained from the analysis of another PDC case (PDC-4) and one Guamanian ALS case with abundant TFGs (data not shown). No insoluble tau was extracted from the frontal brain of a Guamanian control. Accumulations of both 3R and 4R tau isoforms were detected in the white matter of the PDC cases. However, when compared with tau in the gray matter, the levels of 4R tau isoforms were high and the levels of 3R tau isoforms were very low, which was different from those in the gray matter in which similar levels of 3R and 4R tau isoforms or slightly higher levels of 3R tau isoforms were detected. Furthermore, the 4R tau band pattern after dephosphorylation was most obvious in cases in which TFGs were abundant in the white matter. These results suggest that the TFGs in the white matter in the patients with PDC were composed predominantly of 4R tau isoforms.

Sarkosyl-insoluble tau from the white matter of AD brains consisted of a triplet of apparent molecular mass 60, 64, and 68 kDa, which resolved into 6 bands after dephosphorylation (data not shown), indicating that in AD, the tau isoforms deposited in the white matter (mostly in axons) were the same as those deposited in the gray matter, although there was far less of the pathologic tau in the white matter than in the gray matter.
DISCUSSION

TFGs are novel and unique tau-positive inclusions that we observed in the frontal white matter of 86% of the patients with PDC examined here. No TFGs were found in the brains of patients with myotonic dystrophy, Pick disease, or AD.

Furthermore, only a few of the Guamanian controls and Guamanian patients with ALS with many NFTs also exhibited TFGs. Globe-shaped and tau-positive inclusions like TFGs have never been described previously, although other tau-positive inclusions have been reported. Immunoelectron microscope observations revealed that putative TFGs are tau-positive structures that take the shape of granules with a diameter of 20 to 30 nm (including the DAB coating). The PDC white matter stained with Klu¨ver-Barrera and Bodian did not mark a significant change in the stainability even in cases with many TFGs, despite atrophy of the white matter TFGs might relate to this peculiar degeneration of the PDC white matter. We became interested in whether the presence of TFGs had a connection to the atrophy of white matter in the PDC brain.

Immunofluorescence double labeling of TFGs, observed with the aid of confocal scanning microscopy, revealed no
colocalization of GFAP and tau staining or of phosphorylated neurofilament and tau staining on these structures. Therefore, it is unlikely that TFGs originate from either astrocytes or axons. TFGs that were closely associated with the outer layer of the myelin sheath were occasionally bordered by the nucleus of what appeared to be oligodendroglia. It is thus likely that TFGs are derived from oligodendroglia.

Many abnormal tau-positive structures in oligodendroglial cells such as ATs and coiled bodies have been observed in human brains (31–34). These structures are invariably observed in brains with CBD and PSP, but are not specific to neurodegenerative disorders. There are no previous reports of disease-specific oligodendrogial tau-positive inclusions that are a pathologic marker for tauopathies. Numerous tau-positive structures have been observed in the white matter of CBD brains (35, 36), and it is difficult to state categorically that no TFGs exist in the CBD white matter. However, none were observed in the CBD brains that were examined in the present study. TFGs bear a striking resemblance to argyrophilic grains morphologically, but the distribution of argyrophilic grains is quite different from that of TFGs. In AGD, argyrophilic grains are observed in cerebral cortex, amygdala, hypothalamus, and claustrum, but the deep white matter of frontotemporal lobes does not contain argyrophilic grains (37). In this study, tiny thread-like structures were also observed in the vicinity of TFGs in PDC. However, these structures were also detected in brains with CBD, PSP, Pick disease, AGD, and AD.

These structures are therefore not specific to any one tauopathy. Recently, Powers et al reported a case with novel leukoencephalopathy associated with tau deposits primarily in the glia of the white matter (38). In this case, tau-positive structures similar to TFGs were observed mainly in the frontal white matter. However, ultrastructurally, these tau deposits look completely different from TFGs, appearing in the form of straight filaments with a diameter of approximately 10 nm.

In Guam, cases of PDC with many NFTs also had many TFGs in the white matter, whereas in those cases with relatively small numbers of NFTs, TFGs were observed only rarely. Similarly, controls and cases of ALS with few NFTs had a small number of TFGs. Thus, it appears that TFGs only develop when the number of NFTs reaches a certain threshold. However, on further investigation, we found no significant lineal correlation between the presence of TFGs and the number of NFTs and granular hazy astrocytes (6). In addition, NFTs are known to be involved in fibril formation, but we found no fibrils in the TFGs. These results suggest that in Guam PDC brains, the mechanisms of tau deposition underlying the formation of NFTs and TFGs are different.

The presence of TFGs in patients with Guam PDC and Guam ALS does not prove that Guam ALS is a different
with 29 amino acid inserts (3R29N). Recombinant tau isoforms zero amino acid inserts (3R0N), and a 3-repeat tau isoform 29 amino acids inserts (4R29N), a 3-repeat tau isoform with 3 minor bands corresponding to a 4-repeat tau isoform with 4-repeat tau isoform with zero amino acid inserts (4R0N), bands appeared as one major band corresponding to a one minor band of 68 kDa. After dephosphorylation, these of 2 major bands of relative molecular mass 60 and 64 kDa, and antibody, HT-7. HT-7 immunoblotting revealed the presence cases stained with the phosphorylation-independent anti-tau the frontal lobe of parkinsonism–dementia complex of Guam disease from classic ALS or that Guam PDC and Guam ALS represent a single disease entity. The number of TFGs in many patients with Guam ALS was similar to that in nonPDC, non-ALS control subjects. This finding indicates that TFGs are typical features of the general Guamanian population, as is the case with NFTs and granular hazy astrocytes. Moreover, our biochemical analysis revealed that the tau in the white matter of Guam PDC brains is composed of 4R tau. Some previous reports have shown that the tangles present in AD and PDC brains share the same profile when examined immunohistochemically with antihyperphosphorylated tau antibodies, and all 6 tau isoforms have been observed in tangle formation in Guamanian PDC as well as in AD brains (39, 40). A biochemical study of PDC brains in from individuals living on the Kii Peninsula also revealed that the dephosphorylated PHF tau protein is composed of all 6 isoforms (41). Until now, a regional biochemical analysis of tau proteins had not been carried out, and the present study represents the first bio-chemical analysis of the white matter of PDC brains (42–47). The results presented here show clearly that after dephosphorylation, the extracted insoluble tau is composed predominantly of the 4R tau isoforms, and that all 6 isoforms can be found in the frontal cortex (gray matter).

In recent years, it has been shown that the tau in sporadic Pick disease (48, 49) and in hereditary FTDP-17 (L266V) is composed of more than one tau isoform (3R and 4R tauopathies) (50). In the study presented here, it has been demonstrated that in Guamanian PDC, there are 2 distinct patterns of tau isoform composition; all 6 tau isoforms occur in the cerebral cortex and the 4R tau isoforms predominantly occur in the cerebral white matter. This is the first report of the existence of this combination of tau isoforms. Because the 4R tau predominant pattern was most obvious in cases in which TFGs were abundant in the white matter, these 4R tau isoforms are thought to reflect the biochemical characteristics of TFG.

This raises the possibility that tau accumulates in those neurons expressing both 3R tau and 4R tau in the cerebral cortex and that tau builds up in those glial cells expressing 4R tau in the white matter. These glial cells exhibited tau isoform patterns such as 4R0N major, 4R29N, 3R0N, and 4R0N minor. Only one pattern of tau isoform was expressed in any one cell type, and the isoform of accumulated tau was dependent on which cells were involved in the lesions.

In a recent study, “tau-immunoreactive inclusions in glial cells in the white matter” resembling TFGs have been reported in cases with “primary progressive aphasia as the initial manifestation of corticobasal degeneration and unusual tauopathies” (51). Although these globular glial inclusions in the white matter are very similar to TFGs in Guamanian PDC, their size is reportedly larger than that of TFGs. In addition, the Western blotting analysis of total brain homogenates carried out in the present study showed 2 bands of relative molecular mass 68 and 64 kDa, in common with CBD but differing from Guam PDC white matter. It is hoped that these “tau-immunoreactive glial inclusions in the white matter” will be investigated further with the aid of immunoelectron microscopy.

In this study, TFGs were found exclusively in PDC brains and could therefore be a characteristic neuropathologic marker of this disease. The tau isoform in the gray matter (3R + 4R tau) was different from that in the white matter (4R tau) in PDC. This difference is thought to be a function of the cell type from which the tau originated. If this is the case, the question remains as to why particular tau isoforms prevail in the different cell types in any particular brain region and what mechanism underlies this process. The mechanism underlying disease-specific and tau-positive ultrastructural formations should be clarified in accordance with their particular tau isoform.

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