American Journal of Pathology, Vol. 140, No. 6, June 1992

Abnormal Isoform of Prion Proteins Accumulates in the Synaptic Structures of the Central Nervous System in Patients with Creutzfeldt-Jakob Disease

Tetsuyuki Kitamoto, Ryong-Wong Shin, Katsumi Doh-ura, Naoyuki Tomokane, Masayuki Miyazono, Tamaki Muramoto, and Jun Tateishi

From the Department of Neuropathology, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka, Japan

A new method, which enabled the first immunohistochemical documentation of abnormal prion protein (PrP) in all patients with Creutzfeldt-Jakob disease (CJD), was established. This method designated as "hydrolytic autoclaving" revealed punctate PrP^{CJD} stainings around the neuronal cell bodies and dendrites in CJD brains. These punctate stainings were almost identical with that of synaptophysin, suggesting PrP^{CJD} accumulations in the synaptic structures. Subcellular fractionation revealed that prion protein in Creutzfeldt-Jakob disease (PrP^{CJD}) was most concentrated in the synaptosomal fraction. In CJD patients with a long clinical course, synaptophysin immunoreactivity decreased, and synaptic PrP^{CJD} accumulated with a wider distribution. These results suggest that synaptic PrP^{CJD} accumulations might be responsible for the neuronal dysfunction and degeneration in CJD. (Am J Pathol 1992, 140:1285–1294)

Creutzfeldt-Jakob disease (CJD), kuru, and Gerstmann-Sträussler syndrome (GSS) are transmissible neurodegenerative diseases in humans, whereas scrapie is a similar disorder in animals. These diseases are caused by slow infectious pathogen-designated prions.[1] Previous immunohistochemical techniques, even after the formic acid enhancement,[2] revealed positive immunolabeling of kuru plaques in patients with GSS and small prion protein (PrP) accumulations in CJD patients with a long clinical course, but not in patients with a short clinical course.[3]

During the past 5 years, the detection of PrP^{CJD} was performed using Western blots after limited proteinase K digestion.[4,5] This procedure made it feasible to detect not only PrP^{CJD}, but also normal cellular isoform of PrP (PrP^{C}) originating from a single-copy chromosomal gene.[6,7] Using Western blotting, we detected PrP^{CJD} in almost all patients with CJD, and mouse PrP^{CJD} in the CJD-infected mouse brains even in the absence of abnormal PrP deposits.[8] Therefore, demonstration by previous immunohistochemical methods still has its limitations. Recently, three different groups reported the immunohistochemical demonstration of PrP^{C} in neurons using specific fixations.[9–11] With these fixations, abnormal accumulations of scrapie form PrP (PrP^{Sc}) were also documented in the neuropils.[9,10] However, the distribution of PrP^{CJD} in human CJD patients still remains to be established, and the precise subcellular localization of PrP^{CJD} needs to be determined.

Recently, Serban et al[12] found that PrP^{Sc} immunoreactivity was enhanced by its exposure to protein denaturants such as chaotropic salts, NaOH, boiling in sodium dodecyl sulfate (SDS), and formic acid. One of these protein denaturants provided the *in situ* detection of PrP^{Sc} in scrapie-infected cell lines.[13] We applied various protein denaturants to formalin-fixed tissue sections to enhance PrP^{CJD} immunoreactivity.[14] Some of these chemical denaturants were found to enhance kuru plaque stainings, however, structures that were newly documented by PrP immunostainings were not present. We found that hydrolysis, which used autoclave procedures on tissue sections, strikingly enhanced the PrP immunoreactivity *in situ*. This new approach documented the synaptic accumulation of PrP^{CJD} in patients with CJD.

Supported by grants (02454245, 03454171) from the Ministry of Education, Science, and Culture, the Ministry of Health and Welfare, the Science and Technology Agency, and the Foundation for Advancement of Clinical Medicine.

Accepted for publication December 30, 1991.

Address reprint requests to Dr. Tetsuyuki Kitamoto, Department of Neuropathology, Neurological Institute, Kyushu University 60, Fukuoka 812, Japan.

Materials and Methods

Plasmid Construction and Purification of Fd-PrP Fusion Protein

The plasmid designated pFd-PrP is based on the prokaryotic vector pFd-APP 770, a derivative of pFd-APP 695,[15] and carries the *lac* promotor and operator in front of the *lac* Z gene. The plasmid pUC HPrP-M, which codes for the entire human PrP into the *Eco*RI site of pUC 19,[16] was digested with *Hae*II and *Hind*III, and blunted with T4 polymerase. The resulting 774 bp fragment, including residues 25–253 of human PrP, was ligated into the SmaI site of the pUC 19 for arranging the frame. The resulting plasmid pUC HPrP-M′ was digested with *Kpn*I and *Sac*I. The resulting 733 bp fragment was ligated in the vector fragment of pFd-APP 770 which was digested with *Kpn*I and *Sac*I. The plasmid pFd-PrP was transformed in *E. coli* BB 1.8.[15] The fusion protein was purified from the inclusion body fraction by preparative SDS-polyacrylamide gel electrophoresis (PAGE) and electroelution. The identity of the purified protein was confirmed by Western blot analysis using prion protein antibodies (Figure 1). The typical yield from cells of 500 ml culture grown for 10 hours in the presence of 1 mmol/l Isopropyl-B-D(−)-thiogalactopyranoside (IPTG) was 2–5 mg of pure Fd-PrP fusion protein.

Antibodies

In this study, we prepared three antibodies against human PrP. We previously described antiserum against kuru plaque core (KPC) purified from a patient with GSS.[3] We applied affinity purification using formyl-cellulofine gel (Chisso Chemicals, Japan) for the KPC antiserum. Affinity-purified antibody, designated anti-KPC, was used in this study. The second antibody against N-terminal domain of PrP was prepared as described.[17] We prepared affinity-purified antibody, designated anti-PrP-N, using N-terminal peptide coupled to formyl-cellulofine gel. The third antibody is a newly generated polyclonal antibody against the Fd-PrP fusion protein. A rabbit was immunized with the Fd-PrP fusion protein (about 500 μg), which was emulsified with complete Freund's adjuvant. After serial boosters (4 times), the antibody titer was checked to immunostain kuru plaques. We also prepared the affinity-purified antibody, designated anti-Fd-PrP.

To check the specificity, we performed a preabsorption study using anti-PrP-N. The anti-PrP-N was diluted 1:200 (0.5 μg/ml) in 2 ml of 5% nonfat milk, 25 mmol/l Tris-HCl (pH 7.6), 0.05% Tween 20, 0.5 mol/l NaCl containing 200 μg of PrP-N peptide or 2 mg of Fd-PrP inclusion bodies. After overnight incubation at 4°C, the solution was centrifuged at 10,000*g* for 30 minutes. The supernatants were used for immunohistochemical staining. We also used anti-synaptophysin antibody (anti-SYP, DAKO, Denmark).

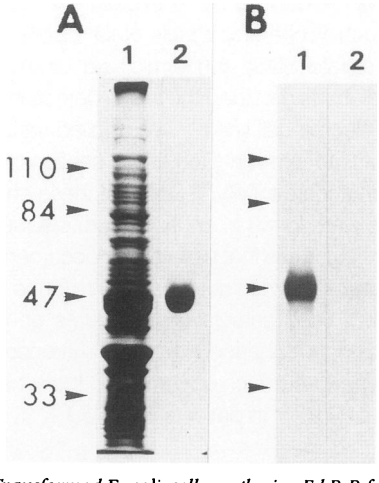

Figure 1. *Transformed* E. coli *cells synthesize Fd-PrP fusion protein. The* E. coli *was transformed with plasmid pFd-PrP, induced with IPTG, and grown for 10 hours at 37°C. Inclusion body fraction was analyzed by SDS-PAGE (10% acrylamide) and visualized with Coomassie brilliant blue (***A**, *Lane 1). 50 kd band was a prominent additional protein compared with the nontransformed* E. coli *(data not shown). The electroeluted fusion protein was analyzed in parallel (***A**, *Lane 2). The eluted Fd-PrP fusion protein was analyzed by Western blots using KPC antiserum (***B**, *Lane 1) or omission of the primary antibody (***B**, *Lane 2).*

Immunohistochemical Analysis Using a New Pretreatment—Hydrolytic Autoclaving

We studied formalin-fixed, paraffin-embedded tissue sections from 50 patients with CJD, and 40 patients with other neurologic disorders (Alzheimer's disease, senile dementia, spinocerebellar degeneration, Parkinson's disease, progressive supranuclear palsy). All available paraffin blocks were cut into 7-μm sections.

We established a novel pretreatment, called "hydrated autoclaving," to enhance the immunoreactivity of tau protein.[18] However, this hydrated autoclaving exerted only limited enhancement for cerebral amyloid compared with the formic acid pretreatment.[2] In search of a more effective enhancing method, we modified the hydrated autoclaving by adding hydrolytic agents, especially HCl, into distilled water. After deparaffinization, endogenous peroxidase was blocked by incubation in methanol containing 0.3% H_2O_2 for 30 minutes. The sections were autoclaved at 121°C for 10 minutes in a stainless-steel vessel filled with 1, 3, 10, or 30 mmol/l HCl in distilled water to immerse the sections. After the pressure in the autoclave had decreased to the atmospheric pressure and the temperature had decreased to 60°C, the sections were taken out and washed with tap water and 50 mmol/l Tris-HCl (pH 7.6). The sections were incubated

with anti-KPC (0.5 μg/ml), anti-PrP-N (0.5 μg/ml), or anti-Fd-PrP (0.5 μg/ml) diluted in TTBS (25 mmol/l Tris-HCl [pH 7.6], 0.05% Tween 20, 0.5 mol/l NaCl) containing 5% nonfat milk at 4°C. The following steps were performed with the unlabeled antibodies biotin-streptavidin method (ICN Immunobiologicals, Costa Mesa, CA) according to the manufacturer's instructions. Diaminobenzidine was used to develop the color.

Preparation of the Subcellular Fractions

We used brain tissues from two patients with CJD and from one patient with systemic amyloidosis. These CJD patients had a short clinical course (3 or 7 months). We used the modified method to purify the fraction of microsome, synaptosome, and synaptic vesicles.[19–21] The cerebral cortex (wet weight 5 g) was homogenized in buffered sucrose (5 mmol/l Hepes [pH 7.4], 320 mmol/l sucrose). The homogenates were centrifuged at 800*g* for 10 minutes. The supernatant was centrifuged at 10,000*g* for 20 minutes. The pellets (P_{10}) were processed to purify either the synaptosome or synaptic vesicles, and the supernatant (S_{10}) was centrifuged at 100,000*g* for 60 minutes to purify the microsome. For microsomal purification, the pellet (P_{100}) was suspended in the buffered sucrose, and the suspension was overlaid onto sucrose gradient (stepwise gradient; 0.32, 0.6, and 1.3 mol/l sucrose). The gradient was centrifuged at 95,000*g* for 90 minutes. The smooth microsomal fraction was recovered at the 0.6 mol/l/ 1.3 mol/l sucrose interface. For the purification of synaptic vesicles and synaptosome, the P_{10} was resuspended in the buffered sucrose, and was centrifuged at 10,000*g* for 20 minutes. The pellet (P_{10-2}) was resuspended in 4 ml of the buffered sucrose. We added 50 ml of ice-cold distilled water into this suspension (hypotonic shock), homogenized briefly, and left the lysate on ice for 30 minutes. The lysate was centrifuged at 22,000*g* for 20 minutes. The supernatant was centrifuged at 165,000*g* for 120 minutes. The final pellet was designated as the synaptic vesicle-rich fraction. For the purification of synaptosome, the P_{10-2} was resuspended in the buffered sucrose, and the suspensions were overlaid onto sucrose gradient (stepwise gradient; 0.32, 1.0, and 1.2 mol/l sucrose). The gradient was centrifuged at 95,000*g* for 90 minutes. The synaptosomal fraction was recovered at the 1.0 mol/l/ 1.2 mol/l sucrose interface. To check the purity of each fraction, we examined under an electron microscope (Hitachi H 7000, Japan) operated at 75 kV.

To detect the PrP^{CJD}, the subcellular fractions purified from 2.5 g tissue were resuspended and sonicated in 2 ml of 50 mmol/l Tris-HCl (pH 7.6), 2% Sarkosyl, 0.5 mol/l NaCl. We added 5 μg of Proteinase K (E. Merck, Germany) into the suspension, and incubated at 37°C for 60 minutes. The digested samples were centrifuged at 411,000*g* for 60 minutes. The final pellets were analyzed by Western blotting[22] and by negative staining for electron microscopic examination.[23] A reflectance densitometric measurement of the intensity of staining and the width of the immunoreactive bands on Western blot membrane was obtained with a Dual Wavelength TLC Scanner (CS-930, Shimazu, Japan).

Double Immunofluorescence

For double immunofluorescent assay[24] with anti-SYP and anti-PrP, we used tissue sections from CJD patients with a short clinical course. After pretreatment with the hydrolytic autoclaving, the sections were blocked with TTBS containing 5% nonfat milk for 30 minutes and were incubated for 48 hours with anti-SYP (5 μg/ml) and anti-PrP-N (10 μg/ml) was diluted with TTBS containing 3% bovine serum albumin (BSA) at 4°C. After washing with TTB (0.1% Triton X-100, 50 mmol/l Tris [pH 7.6]), the sections were incubated overnight with fluorescein-linked donkey anti-rabbit IgG (1:50, Amersham, UK) and biotinylated anti-mouse IgG (ICN Immunobiologicals) at 4°C. After washing with TTB, they were then incubated for 2 hours with Texas-Red conjugated streptavidin (1:100 in TTBS containing 3% BSA, Amersham) at room temperature. After washing with TTB, the sections were mounted in glycerol, and examined by a Zeiss Axiophoto microscope (Germany).

Results

Effect of Hydrolytic Autoclaving on PrP Immunoreactions

In formalin-fixed paraffin-embedded tissue sections from CJD patients with a long clinical course, a few PrP deposits were documented positively with PrP antibodies (anti-KPC, anti-PrP-N, or anti-Fd-PrP) without applying hydrolytic autoclaving. When the sections were treated with hydrolytic autoclaving, diffuse gray matter stainings were noted (Figure 2). These diffuse stainings were widely distributed through the gray matter of the central nervous system. The diffuse gray matter stainings were detected in all the CJD patients examined, but not in 40 patients with other neurologic diseases.

At first, we evaluated the optimal concentration of HCl for hydrolysis of tissue sections. We examined concentrations of HCl at 0, 1, 3, 10, and 30 mmol/l. The best efficacy was obtained at a concentration of 3 mmol/l HCl in most of the cases. In two different situations, however, autoclaving at 3 mmol/l HCl did not provide efficacy; in one case, autoclaving at this concentration was so ex-

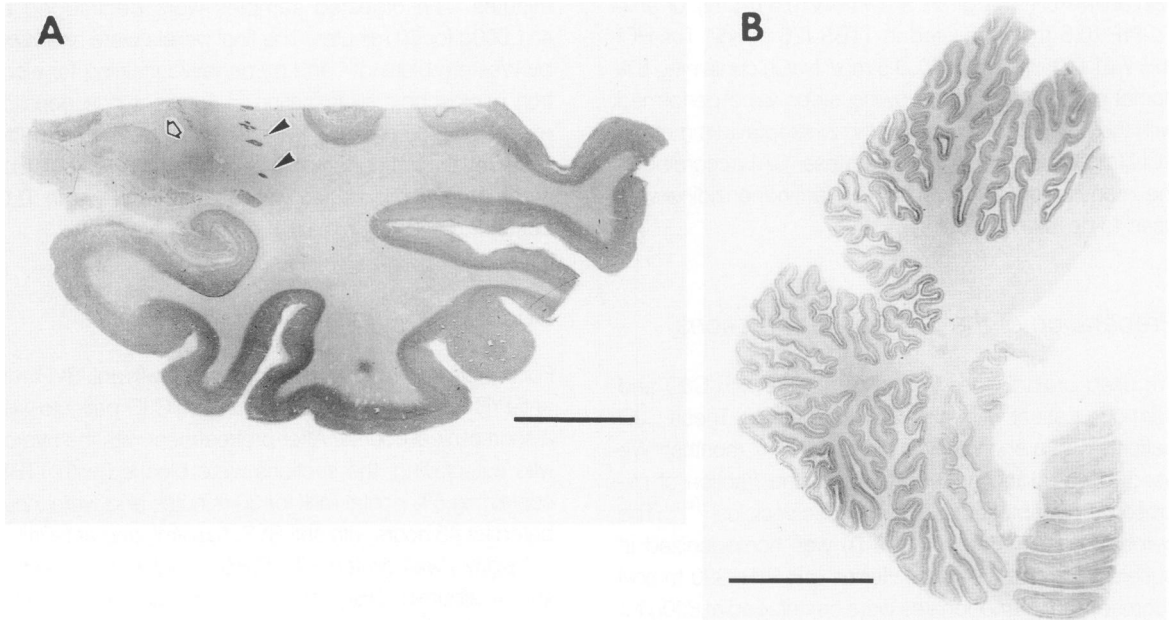

Figure 2. *Macroscopic observations of PrP immunostainings after hydrolytic autoclaving. Anti-Fd-PrP revealed diffuse gray matter stainings after hydrolytic autoclaving. In the temporal lobe including hippocampus of a CJD patient* **(A)**, *diffuse immunolabelings are observed in the whole gray matter areas such as the cortex, caudal part of putamen (solid arrows), lateral geniculate body (open arrow), whereas white matter is not immunolabeled. In the vermis of the cerebellum of another CJD patient* **(B)**, *diffuse immunolabelings are also observed entirely in the molecular layer and the granular cell layer. Bar = 1 cm.*

cessive that it damaged the morphology of the tissue and resulted in the ineffective enhancement of PrP immunoreactions. We, therefore, reduced the concentration to 1 mmol/l HCl in such a case. In the second case, autoclaving at 3 mmol/l HCl was insufficient to enhance PrP immunoreactions. In such a case, we raised the concentration of HCl to either 10 mmol/l or 30 mmol/l. Thus, we selected the concentration of 3 mmol/l HCl at first, and when it turned out to be ineffective we changed the concentration to either decrease or increase HCl.

To evaluate the hydrolytic activities during autoclaving, we carried out Western blot analysis using the electroeluted Fd-PrP fusion protein (Figure 3). As the concentration of HCl increased, the hydrolytic activities also increased. Finally, autoclaving at 30 mmol/l HCl hydrolyzed the Fd-PrP fusion protein into small peptides that were not immunolabeled by the anti-KPC antibodies. Excessive hydrolysis of the peptide bond diminished the PrP immunoreactions on the immunoblots, as with the damaged tissue sections.

Newly Recognized Structures Containing PrP^{CJD}

Although we could not detect any PrP^{C} immunoreactions in the 40 control patients with other neurologic diseases, kuru plaques in GSS patients were strongly immunostained after hydrolytic autoclaving. However, the most conspicuous findings obtained after hydrolytic autoclaving were diffuse gray matter stainings in macroscopic observations. Microscopic observations, especially in CJD patients with a short clinical course, helped to visualize the precise localization of PrP^{CJD}. CJD patients with a long clinical course had damaged structures in their brains, so-called "status spongiosus." Only dot-like stainings, which were similar to the diffuse neuropil stainings in scrapie, were distributed diffusely throughout the gray matter in the central nervous system.[9,10] However, using the tissue sections from CJD patients with a short clinical course, we were able to establish a characteristic staining pattern in the globus pallidus (Figure 4). Immunostained structures were composed of individual small punctate stainings which were suggestive of synaptic bouton staining, and appeared like so-called 'woolly fibers' revealed by immunostainings with the marker of axon terminals.[25,26] To compare with PrP immunostainings, we used anti-SYP as the marker of synaptic vesicles. Anti-SYP immunolabeled small punctate structures, and these punctate stainings built up 'woolly fibers' in the globus pallidus (Fig. 4). The same structures were reproduced by all our 3 antibodies against human PrP. In the cerebral cortex, the punctate stainings with anti-PrP were seen around the cortical neurons (Fig. 5). Anti-PrP did not

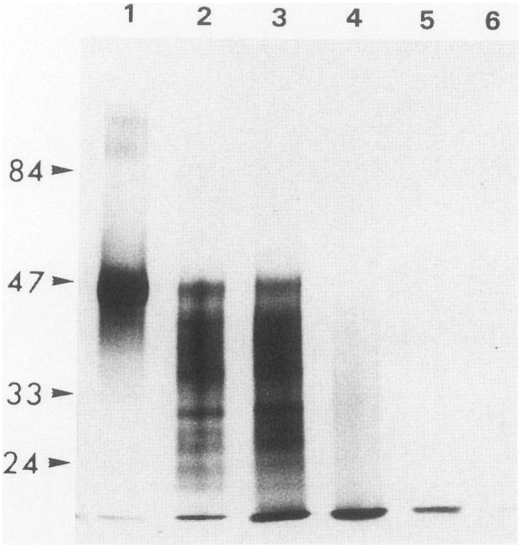

Figure 3. *Effect of hydrolytic autoclaving on Fd-PrP fusion protein on Western blot. The electroeluted fusion protein was dialyzed against distilled water and lyophilized. The lyophilized fusion protein was dissolved in 1% SDS solution in distilled water and was treated with the following procedures. Fd-PrP in Lane 1 was boiled ordinarily at 100°C for 10 minutes. To samples in Lane 2 to 6, we added 0, 1, 3, 10, or 30 mmol/l HCl at final concentration, respectively, and autoclaved them. The samples were analyzed by SDS-PAGE (10% acrylamide) and Western blotting with KPC antiserum. Fd-PrP fusion protein is hydrolyzed increasingly according to increasing concentration of HCl. In Lane 5 (10 mmol/l HCl), Fd-PrP exhibits immunoreactions only at the dye front. In Lane 6 (30 mmol/l HCl), Fd-PrP is thoroughly hydrolyzed into peptides and is no longer recognized immunochemically.*

react with cytoplasm of neurons. In the cerebellum, anti-PrP revealed fine dotted stainings in the molecular layer, and coarse dotted stainings in the granular cell layer (Fig. 5). These fine and coarse dotted stainings were also stained with anti-SYP, and the coarse dotted stainings may correspond to the cerebellar glomeruli which are composed of axon terminal of mossy fibers. In the brain stem and the spinal cord, these punctate stainings were also documented in almost all subcortical nuclei, but the staining intensities varied from case to case. Even after hydrolytic autoclaving, no convincing immunostainings were observed in either the astrocytes, oligodendrocytes, ependymal cells or vascular walls of CJD patients.

To check the specificity of immunoreaction, we prepared and used three different antibodies against human PrP, and performed negative control studies including preabsorption, omission of the primary antibody, and immunostainings using purified IgG (0.5 μg/ml) from preimmune sera. The PrP^{CJD} positive structures shown here were equally documented by three PrP antibodies, and were abolished by the control studies (Figure 6).

In our preliminary study, CJD patients with a long clinical course had dot-like stainings distributed diffusely throughout the gray matter. The morphology of the brain tissues was markedly damaged due to the prolonged course of the disease, and the fine punctate PrP^{CJD} stainings around the corical neurons could no longer be observed. In a CJD patient with a long clinical course, severe neuronal loss was observed in the cerebral neocortex and the cerebellar granular cell layer. In such patients, SYP immunoreactivity was moderately to markedly decreased due to neuronal cell death. However, we

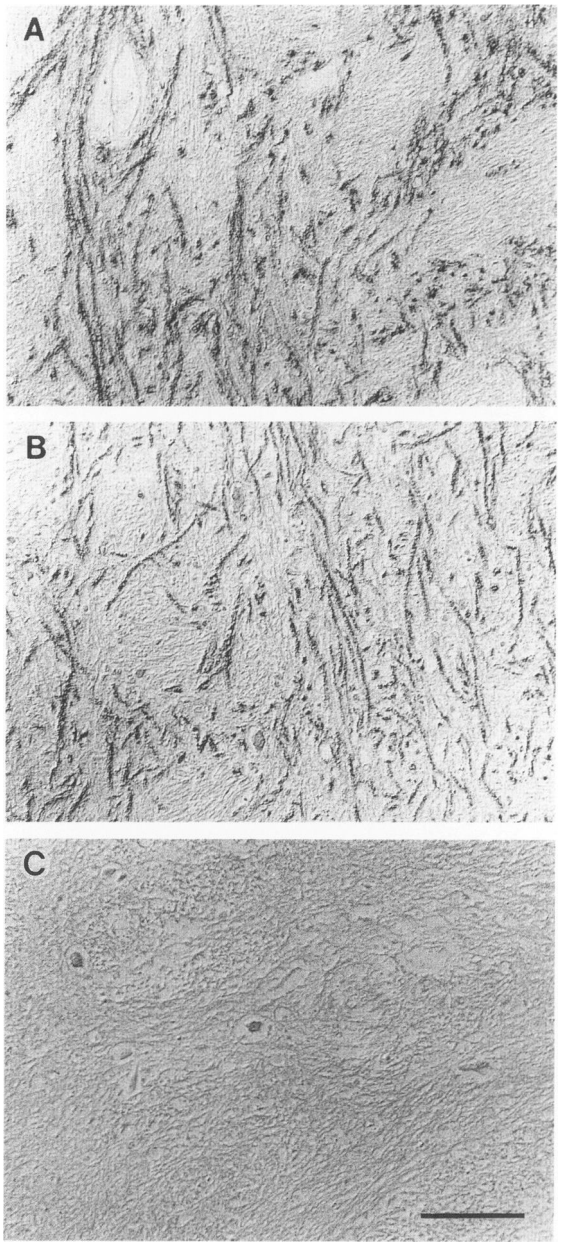

Figure 4. *Newly revealed PrP^{CJD} structures in the globus pallidus. In the globus pallidus of a CJD patient* **(A)**, *anti-PrP-N reveals the structures designated "woolly fibers," which are composed of small punctate PrP stainings. These punctate stainings are seen mainly on the dendrites, and sometimes on the neuronal cell bodies. In the same patient* **(B)**, *anti-SYP also reveals the same structures in the globus pallidus (nonserial section). In a patient with Alzheimer's disease* **(C)**; *however, anti-PrP-N does not show any immunoreactions in the globus pallidus. Bar = 100 μm.*

detected punctate PrP^{CJD} or SYP stainings in the hippocampus and the brain stem nuclei, where the neuronal structures were well preserved in spite of the prolonged disease course (Figure 6).

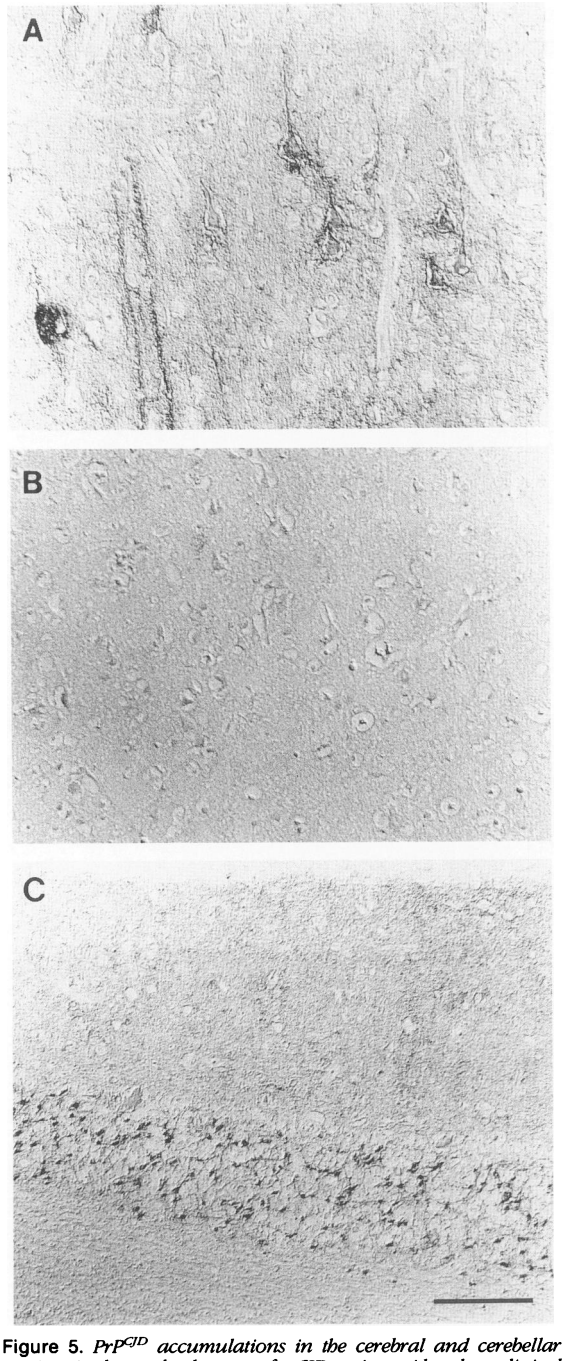

Figure 5. *PrP^{CJD} accumulations in the cerebral and cerebellar cortices. In the cerebral cortex of a CJD patient with a short clinical course* (A), *anti-PrP-N documents punctate stainings around the cell bodies and dendrites of cortical neurons. While in the cerebral cortex of a patient with Alzheimer's disease, the documentation of PrP immunoreactions was not obtained* (B). *In the cerebellar cortex of a CJD patient* (C), *fine punctate stainings are observed in the molecular layer, and coarse dotlike stainings are seen in the granular cell layer. These punctate stainings were sometimes seen around the cell bodies of Purkinje cell. Bar = 100 μm.*

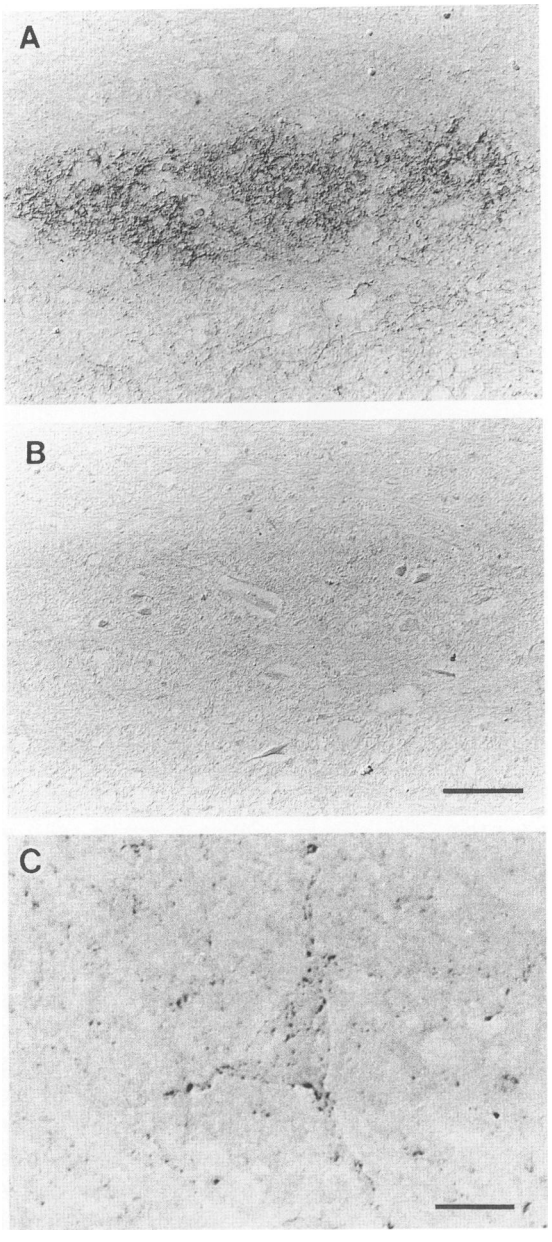

Figure 6. *Synaptic PrP stainings in CJD patients with a long clinical course. Anti-PrP-N immunostainings reveal fine punctate structures in the inferior olive nucleus of a CJD patient with a long clinical course* (A). *The absorption of anti-PrP-N with synthetic PrP-N peptide abolishes these synaptic stainings in the serial section* (B). *In another CJD patient with a long clinical course, anti-KPC reveals fine punctate stainings around the cell body and dendrites of a neuron in CA 4 region of the hippocampus* (C). *Bar = 100 μm in* (A) *and* (B). *Bar = 20 μm in* (C).

For a precise comparison of the immunolabeled structures revealed by anti-PrP and those by anti-SYP, we performed a double-immunofluorescent analysis. Unfortunately, lipofuscin granules in neurons had endogenous fluorescent activity, and interfered in the precise analysis around neurons. Therefore, we examined globus pallidus and substantia nigra in which woolly fibers

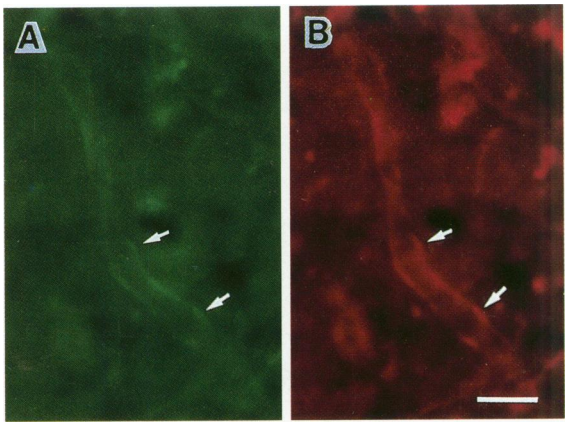

Figure 7. *Double immunofluorescent study with anti-PrP-N and anti-SYP. PrP immunoreactions are documented with FITC (green), and SYP reactions with Texas Red (red). On one woolly fiber in the globus pallidus of a CJD patient, PrP positive reactions* **(A)** *are colocalized with SYP reactions* **(B)**. *Arrows show the colocalized synaptic structures. Bar = 10 μm.*

were observed in anti-PrP or anti-SYP immunostainings. Double immunofluorescein showed the colocalization of the PrP^{CJD} and SYP immunoreactivities on the woolly fibers (Figure 7).

Subcellular Fractions to Detect PrP^{CJD} and PrP^{C}

To obtain biochemical information about the synaptic structures, we examined the synaptosome, synaptic vesicle-rich, and microsome fractions. Previous studies reported that microsomal fractions,[27] or microsomal and synaptosomal fractions[28] contained PrP^{Sc} in scrapie-infected animals. Compared with the amyloid fibril purification method,[29] amyloid fibrils may be copurified in the microsomal fractions. Therefore, we performed additional sucrose gradient centrifugation to get smooth microsomal fractions and to avoid amyloid fibril contamination. In case of synaptic vesicle purification, we could not get enough yields to perform Western blot analysis in the final step reported by Huttner et al.[20] Therefore, we used the intermediate step of synaptic vesicle-rich fractions.

Concerning the yields of the synaptosomal fractions, we got 1000 to 1200 μg protein/g tissue from a non-CJD control subject, 750 μg/g from a CJD patient with a 3-month clinical course, and 150 μg/g from another CJD patient with a 7-month duration. By Western blotting, we analyzed the concentration of total PrP (PrP^{CJD} and PrP^{C}) in each subcellular fraction. We applied 10 μg protein of the subcellular fraction to each well of the gel. To detect PrP^{CJD}, we also applied proteinase-digested samples, which were originally derived from each 10 μg protein of the subcellular fraction. The synaptosomal fraction was found to have the highest concentration of PrP^{CJD} (Figure 8). In protease-digested fractions from the non-CJD case, we were not able to identify any positive immunoreactions. To compare the PrP concentration in each fraction of the CJD patient, we carried out a reflectance densitometric measurement (Figure 8). We measured the immunolabeled bands of 34–36, 29–33, 24–27, and 19–21 kd in each subcellular fraction, and the bands of 27–31, 23–35, and 19–21 kd in each proteinase-digested fraction. The ratio of the PrP^{CJD} to the total PrP was 67% in the synaptosome, 35% in the microsome, and 16% in the synaptic vesicle-rich fraction.

To examine the purity of each subcellular fraction, we observed the fractions under electron microscopic examination (Figure 8). The subcellular fractions exhibited compatible structures as reported previously,[19,21] and we recognized many prion rods in the proteinase-digested synaptosomal fraction from the CJD patients.

Discussion

We have achieved the *in situ* immunodetection of PrP^{CJD} in all patients with CJD. Our results show that PrP^{CJD} accumulates mainly in the synaptic structures of neurons throughout the central nervous system. These observations suggest that synaptic PrP^{CJD} accumulations might be responsible for both the neuronal dysfunction and cell death that are seen in CJD patients.

Protein Denaturation and Immunoreactivity of PrP^{CJD}

We and other groups have reported that the extensive denaturation of PrP^{CJD} or PrP^{Sc} was necessary to promote the binding of antibodies to the epitopes of PrP.[2,12,13,30] Although a number of studies have indicated that antibodies made against denaturated proteins react more readily with denaturated antigens, the extreme increase in PrP^{CJD} immunoreactivity revealed here was unexpected. The mechanism of protein denaturation during hydrolytic autoclaving could thus be speculated by the previous inactivation studies of enzymes.[31] Under

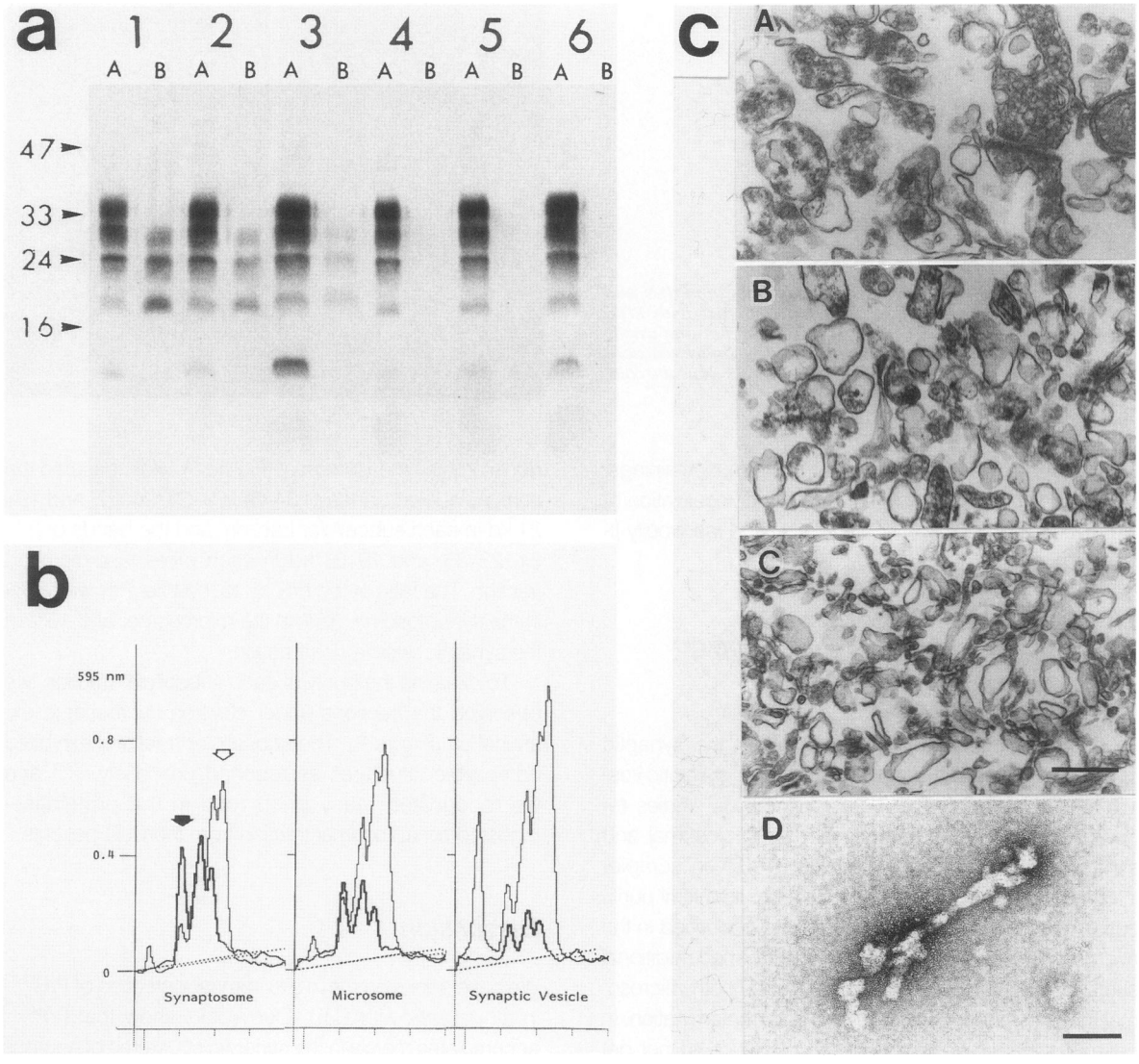

Figure 8. *Subcellular localization of PrP^{CJD} and PrP^{C}.* **a:** *Western blot analysis with each subcellular fraction. Lane 1 corresponds to synpatosome, Lane 2 to microsome, and Lane 3 to synaptic vesicles, all of which were purified from a CJD patient with a 3-month duration. Lane 4 corresponds to synaptosome, Lane 5 to microsome, and Lane 6 to synaptic vesicles, which were purified from a non-CJD patient. In each Lane A, we applied 10 μg protein from each subcellular fraction. In each Lane B, we applied detergent-insoluble, proteinase-resistant samples that were originally derived from 10 μg of subcellular fractions to detect PrP^{CJD}.* **b:** *Densitometric measurement of total PrP and PrP^{CJD} in subcellular fractions purified from a CJD patient. We compared the intensity of the immunoreactive bands on Western blots using a reflectance densitometer. Thin lines are corresponding to the absorbance of Lane A (total PrP) in* **(a)**, *and thick lines to Lane B (PrP^{CJD}). Solid arrow shows the peak corresponding to 19–21 kd band, and open arrow corresponding to 34–36 kd.* **c:** *Electron microscopic analysis of subcellular fractions from a CJD patient. Synaptosome (A), microsome (B), synaptic vesicles (C), prion rod (D). We could find many axon terminals in (A), smooth microsomes in (B). In (C), the synaptic vesicle-rich fraction contains the contamination of smooth microsome. Because of low protein yields in the synaptic vesicle purification, we had to analyze the intermediate step of the purification method (Materials and Methods); (D) shows prion rods in detergent-insoluble, proteinase-resistant fraction from synaptosome purified from a CJD patient. Bar = 300 nm in (A), (B) and (C). Bar = 100 nm in (D).*

acidic condition at high temperatures, enzyme inactivation is caused by hydrolysis of peptide bonds at Asp residues, deamidation of Asn or Gln residues, and the destruction of disulfide bonds including β-elimination.[32] Western blot analysis after hydrolytic autoclaving showed that Fd-PrP fusion protein was hydrolyzed increasingly according to the concentration of HCl. This hydrolytic activity on the fusion protein correlated well with its enhancing effect on *in situ* PrP^{CJD} immunoreactivity. Therefore, hydrolytic autoclaving may alter the primary structures (peptide bond hydrolysis, deamidation, etc.) of PrP *in situ*. We could not detect any PrP^{C} immunoreactions in non-CJD cases. These results suggest that the hydrolytic procedure may be too drastic for PrP^{C} immunoreactivity to be revealed. Under a milder hydrolytic condition, however, we did not reveal PrP^{C} immunoreaction constantly.

Therefore, the demonstration of PrP^{C} immunoreactivity may be also dependent on the fixation conditions.

Synaptic Localization of PrP^{CJD}

Synaptic staining of PrP^{CJD}, as well as the highest concentration of PrP^{CJD} in the synaptosomal fractions, suggest that PrP^{CJD} is accumulated in the synapses during the disease process. In spite of the decreased SYP immunoreactivity and the low yields of synaptosomal fractions in a CJD patient with a 7-month clinical course, they still had diffusely distributed dotlike PrP immunoreactivities. Therefore, the majority of the PrP^{CJD} positive synaptic structures corresponded more closely to that of damaged synapses rather than to the intracellular accumulations of PrP^{CJD}. Even in CJD patients with a long clinical course, the fine punctate synaptic stainings were revealed with PrP and SYP immunostaining in their hippocampus and brain stem nuclei. It is well known that the hippocampus and brain stem nuclei are regions resistant to the disease process. These data suggest that the neuronal dysfunction and cell death may follow the degeneration of synapses with results from abnormal accumulations of PrP^{CJD}.

Recently, dystrophin molecules were observed in the postsynaptic regions of the central nervous system.[33] Anti-dystrophin immunostainings also revealed synaptic punctate stainings in a similar manner as anti-SYP (presynaptic marker). The precise localization of PrP^{CJD} in the synaptic structures will be important, and the higher resolution achievable by immunoelectron microscopic examination may help resolve this issue. At present, however, these synaptic stainings are revealed only by hydrolytic autoclaving, and this pretreatment destroys the fine morphologic structures at the electron microscopic level. We still can not identify in which side of the synapsis, presynaptic or postsynaptic regions, PrP^{CJD} accumulates. However, previous reports have put forth the hypothesis of PrP^{CJD} localization.[34–36] After intraocular inoculation with either scrapie or CJD agent, scrapie infectivity and degenerative vacuolations were initially localized within the contralateral superior colliculus in mice.[34,36] In some scrapie mice, the visual cortex was also involved unilaterally in the late stage.[35] Therefore, it is becoming clear that a major route of the agent spread involves neuronal pathways probably via axonal and trans-synaptic infection. They also reported that the infectious agent accumulated in the target region (superior colliculus) rather than the axonal portion (optic nerve). Their findings correlate well with the synaptic PrP^{CJD} accumulations reported here, and suggest that the site of accumulations may be the axon terminals. Hydrolytic autoclaving revealed PrP^{CJD} accumulations in the gray matter, not in the white matter, or if present then only slightly. Our immunohistochemical results correspond with these scrapie infectivity studies.

Previously, a major obstacle for studying the pathogenesis of CJD arose from the inability to recognize PrP^{CJD} *in situ*. However, hydrolytic autoclaving provides a clue to understanding local events in the central nervous system with CJD. Synaptic PrP^{CJD} accumulation may explain a loss of normal neuronal functions, which results in dementia as seen in CJD patients. We found that PrP^{CJD} accumulation may play a central role in the pathogenesis of CJD.

Acknowledgments

The authors thank Dr. Kosaka (Kyushu University) for drawing our attention to the synaptic structures; Drs. Beyreuther and Weidemann (University of Heidelberg) for helping with the construction of pFd-APP 770; Dr. Mehraein (University of München) for providing European CJD cases; Drs. Okochi, Sato, Ishibashi, Nagafuchi, Nagara for providing valuable discussion; Miss Hatanaka and Mr. Yoneda for providing technical assistance; and Dr. B. Quinn for reviewing the manuscript.

References

1. Prusiner SB: Novel proteinaceous infectious particles cause scrapie. Science 1982, 216:136–144
2. Kitamoto T, Ogomori K, Tateishi J, Prusiner SB: Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab Invest 1987, 57:230–236
3. Kitamoto T, Tateishi J: Immunohistochemical confirmation of Creutzfeldt-Jakob disease with a long clinical course with amyloid plaque core antibodies. Am J Pathol 1988, 131:435–443
4. Bockman JM, Kingsbury DT, McKinley MP, Bendheim PE, Prusiner SB: Creutzfeldt-Jakob disease prion proteins in human brains. N Engl J Med 1985; 312:73–78
5. Brown P, Coker-Vann M, Pomery K, Franko M, Asher DM, Gibbs CJJr, Gajdusek DC: Diagnosis of Creutzfeldt-Jakob disease by Western blot identification of marker protein in human brain tissue. N Engl J Med 1986, 314:547–551
6. Oesch B, Westaway D, Wälkli M, McKinley MP, Kent SBH, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C: A cellular gene encodes scrapie PrP 27–30 protein. Cell 1985, 40:735–746
7. Basler K, Oesch B, Scott M, Westaway D, Wälkli M, Groth DF, McKinley MP, Prusiner SB, Weissmann C: Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 1986, 46:417–428
8. Kitamoto T, Yi R, Mohri S, Tateishi J: Cerebral amyloid in mice with Creutzfeldt-Jakob disease is influenced by the strain of the infectious agents. Brain Res 1990, 508:165–167
9. DeArmond SJ, Mobley WC, DeMott DL, Barry RA, Beckstead JH, Prusiner SB: Changes in the localization of brain

prion proteins during scrapie infection. Neurology 1987, 37:1271–1280
10. Bruce ME, McBride PA, Farquhar CF: Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. Neurosci Lett 1989, 102:1–6
11. Piccardo P, Safar J, Ceroni M, Gajdusek DC, Gibbs CJ,Jr: Immunohistochemical localization of prion protein in spongiform encephalopathies and normal brain tissue. Neurology 1990, 40:518–522
12. Serban D, Taraboulos A, DeArmond SJ, Prusiner SB: Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. Neurology 1990, 40:110–117
13. Taraboulos A, Serban D, Prusiner SB: Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells. J Cell Biol 1990, 110:2117–2132
14. Doi-Yi R, Kitamoto T, Tateishi J: Immunoreactivity of cerebral amyloidosis is enhanced by protein denaturation treatments. Acta Neuropathol 1991, 82:260–265
15. Weidemann A, König G, Bunke D, Fisher P, Salbaum JM, Master CL, Beyreuther K: Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. Cell 1989, 57:115–126
16. Doh-ura K, Tateishi J, Sasaki H, Kitamoto T, Sakaki H: Pro - Leu change at position 102 of prion protein is the most common but not the sole mutation related to Gerstmann-Sträussler syndrome. Biochem Biophys Res Commun 1989, 163:974–979
17. Kitamoto T, Muramoto T, Hilbich C, Beyreuther K, Tateishi J: N-terminal sequence of prion protein is also integrated into kuru plaques in patients with Gerstmann-Sträussler syndrome. Brain Res 1991, 545:319–321
18. Shin RW, Iwaki T, Kitamoto T, Tateishi J: Hydrated autoclave pretreatment enhances Tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. Lab Invest 1991, 64:693–702
19. Gordon-Weeks PR: Isolation of synaptosome, growth cones and their subcellular components. Neurochemistry. Edited by Turner AJ, Bachelard HS. Oxford, IRL Press, 1987, pp 1–25
20. Huttner WB, Schiebler W, Greengard P, De Camilli P: Synapsin I (Protein I), a nerve terminal specific phosphoprotein. III. Its association with synaptic vesicles studies in a highly purified synaptic vesicle preparation. J Cell Biol 1983, 96:1374–1388
21. Lambert M: Isolation of microsomes. Protein Purification Methods. Edited by Harris ELV, Angal S. Oxford, IRL Press, 1989, pp 101–108
22. Kitamoto T, Mohri S, Tateishi J: Organ distribution of protease-resistant prion protein in humans and mice with Creutzfeldt-Jakob disease. J Gen Virol 1989, 70:3371–3379
23. Kitamoto T, Hikita K, Tashima T, Tateishi J, Sato Y: Scrapie-associated fibrils (SAF) purification method yields amyloid proteins from systemic and cerebral amyloidosis. Biosci Rep 1986, 6:459–465
24. Kitamoto T, Muramoto T, Mohri S, Tateishi J: Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J Virol 1991, 65:6292–6295
25. Haber SN, Nauta WJH: Ramifications of the globus pallidus in the rat as indicated by patterns of immunohistochemistry. Neuroscience 1983, 9:245–260
26. Goto S, Hirano A: Synaptophysin expression in the striatum in Huntington's disease. Acta Neuropathol 1990, 80:88–91
27. Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA, Prusiner SB: Separation and properties of cellular and scrapie prion proteins. Proc Natl Acad Sci USA 1986, 83:2310–2314
28. Safar J, Ceroni M, Piccardo P, Liberski PP, Miyazaki M, Gajdusek DC, Gibbs CJ,Jr: Subcellular distribution and physicochemical properties of scrapie-associated precursor protein and relationship with scrapie agent. Neurology 1990, 40:503–508
29. Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC: The characterization of soluble amyloid prepared in water. J Clin Invest 1968, 47:924–933
30. Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H: Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 1987, 61:3688–3693
31. Volkin DB, Klibanov AM: Minimizing protein inactivation. Protein Function. Edited by Creighton TE. Oxford, IRL Press, 1989, pp 1–24
32. Ahern TJ, Klibanov AM: The mechanism of irreversible enzyme inactivation at 100°C. Science 1985, 228:1280–1284
33. Lidov HGW, Byers TJ, Watkins SC, Kunkel LM: Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature 1990, 348:275–278
34. Fraser H: Neuronal spread of scrapie agent and targeting of lesions within the retino-tectal pathway. Nature 1982, 295:149–150
35. Fraser H, Dickinson AG: Targeting of scrapie lesions and spread of agent via the retino-tectal projection. Brain Res 1985, 346:32–41
36. Liberski PP, Yanagihara R, Gibbs CJJr, Gajdusek DC: Spread of Creutzfeldt-Jakob disease virus along visual pathways after intraocular inoculation. Arch Virol 1990, 111:141–147