Establishment of a Stable PrP^{Sc} Panel from Brain Tissues of Experimental Hamsters with Scrapie Strain 263K[1]

BAO-YUN ZHANG*, CHAN TIAN*, JUN HAN, CHEN GAO, QI SHI, JIAN-MING CHEN, HUI-YING JIANG, WEI ZHOU, AND XIAO-PING DONG[2]

State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China

Objective To establish a stable PrP^{Sc} panel from brain tissues of experimental hamsters infected with scrapie agent 263K for evaluating diagnostic techniques of human and animals' prion diseases. **Methods** Thirty brain tissue samples from hamsters intracerebrally infected with scrapie strain 263K and another 30 samples from normal hamsters were selected to prepare 10%, 1%, and 0.5% brain homogenates, which were aliquoted into stocks. PrP^{Sc} in each brain homogenate was determined by proteinase K digestions followed by Western blot assay and partially by immunohistochemistry. Stability and glycoforms of PrP^{Sc} were repeatedly detected by PrP^{Sc}-specific Western blots in half a year and 3 years later. **Results** PrP^{Sc} signals were observed in all 10% brain homogenates of infected hamsters. Twenty out of 30 stocks and 19 out of 30 stocks were PrP^{Sc} positive in 1% and 0.5% brain homogenatesof infected hamsters, respectively. Twenty-seven out of 30 stocks presented three positive bands in 10% brain homogenates, whereas none of 1% and 0.5% homogenates contained 3 bands. The detection of PrP^{Sc}-specific signals stored in half a year and 3 years later demonstrated that the ratio of PrP^{Sc} positive samples and glycoforms was almost unchanged. All normal hamsters' brain homogenates were PrP^{Sc} negative. **Conclusion** A PrP^{Sc} panel of prion disease can be established, which displays reliably stable PrP^{Sc}-specific signals and glycoforms.

Key words: TSE; PrP^{Sc}; Panel; Glycoforms; Stability

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), namely prion diseases, are a group of neurodegenerative diseases[1], including scrapie in sheep, bovine spongiform encephalopathy in cattle, chronic wasting disease (CWD) in deer and elk and Creutzfeldt-Jakob disease (CJD) in humans. These types of diseases have similar and unique clinical and pathological characteristics, e.g. spongiform degeneration in brains, 100% death rate, extremely long incubation but short duration after onset, *etc*. PrP^{C}, the host-encoded glycophosphatidylinositol anchored prion protein, is constitutively expressed in the central nervous system (CNS), which has been proposed to have numerous biological functions. However, the abnormal form of PrP^{C}, PrP^{Sc}, is believed to be an infectious and causal agent for TSE, which not only accumulates in brain tissues of animals or humans with TSE causing cytotoxicity, but also transmits the diseases among homo and heterogenous animals[2].

During the 1980s, an outbreak of a novel form of TSE, bovine spongiform encephalopathy (BSE), occurred in cattle in UK and other European countries, causing hundred thousands of cattle deaths. Shortly afterwards, BSE was confirmed to be able to infect other species, e.g. cats and other ruminants. In 1996, variant CJD (vCJD), a new kind of human TSE, was described in UK and France, which was verified to have been caused by consuming BSE-contaminated food. Up to now, more than 200 individuals have died of vCJD, mostly in Europe, but also in North America and Asia[3]. It was recently

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[2]Correspondence should be addressed to Xiao-Ping DONG. Tel: 86-10-83534616. Fax: 86-10-63564478. E-mail:dongxp238@sina.com

Biographical note of the first authors: Bao-Yun ZHANG, male, born in 1956, majoring in virus morphology and the mechanism of prion diseases; Chan TIAN, female, born in 1980, Ph. D., majoring in immunology and mechanism of prion diseases.

*The first two authors contributed equally to this article.

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reported that vCJD infectious agent can be transmitted among humans by blood transfusion[4]. The outbreaks of BSE and vCJD have caused huge impact not only on stockbreeding and related industries, but also on public health.

At present, the definite diagnosis of animal and human TSE is still based on the detection of PrP^{Sc} or on the observation of special pathological changes in brain tissues that are usually obtained by postmortem. Research and development (R&D) of more sensitive and specific diagnostic techniques for TSE are one of the essential steps to eliminate or eradicate the impacts of TSE. Actually, besides the traditional pathological and immunological methods, several new measurement technologies are being investigated[5-9]. As the R&D processes of other diagnostic techniques, a reliable and well-designed PrP^{Sc} panel is important for the assessment of newly established techniques for TSE.

Although the prion strains from various animal and human TSEs vary each other in some aspects, their immunoreactivities share a high similarity that is possible to set up one panel for TSE of different species. In this study, a PrP^{Sc} panel of brain tissues from experimental hamsters infected with scrapie agent 263K was established, which consisted of 30 brain tissue samples from 263K-infected hamsters and 30 tissue samples from normal hamsters. Each brain tissue sample was prepared in three different concentrations and the presence of PrP^{Sc} in stocks was evaluated. Furthermore, the main immunological and biochemical stabilities of PrP^{Sc} in the stored stocks were assayed three years after establishment of the panel. The work provides reliable experimental and material bases for the evaluation of diagnostic technologies for TSE.

MATERIALS AND METHODS

Preparation of Brain Homogenates

Ten percent brain homogenates were prepared with brain tissues of the hamsters infected with scrapie strain 263K and of normal hamsters in a lysis buffer containing 10 mmol/L NaCl, 10 mmol/L EDTA, 0.5% NP-40, 0.5% sodium deoxycholate and 10 mmol/L Tris, pH 7.4. The homogenates were centrifuged at 400×*g* for 10 min, and supernatants were collected for further assay. The homogenates were diluted at 1:10 and 1:20 with the lysis buffer to generate 1% and 0.5% brain homogenates. All homogenates were aliquoted into stocks and stored at -80 ℃.

Western Blot Assay

Samples were digested by 50 μg/mL proteinase K (PK) at 37 ℃ for 2 h, separated in 15% SDS-polyacrylamide gel (SDS-PAGE) and electronically transferred to PVDF membrane (Immobilon-P, Millipore). After blocked with 5% non-fat milk powder in PBST, the membrane was incubated with 1:4000 diluted PrP-specific monoclonal antibody 3F4 (Dako, Denmark) at 4 ℃ overnight. After washed twice with PBST, the membrane was incubated with 1:4000 diluted horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Boehringer) in PBST at room temperature for 1 h followed by detection of signals with an enhanced chemo-fluorescence detection kit (Amersham-Pharmacia Biotech, USA).

Immmunohistochemical Assay

The brain tissue samples from normal and infected hamsters were subjected to formalin fixation, formic acid treatment and paraffin embedding following the conventional protocols. After dewaxation, paraffin slices were exposed to 4 mol/L guanidinium isothiocyanate at 4 ℃ for 2 h, microwave irradiation (Galanz, WP1000L30-2) in distilled water for 30 min and 3% hydrogen peroxide-methanol for 10 min, respectively. The slices were blocked with normal goat serum for 15 min, incubated with 1:500 diluted mAb 3F4 at 4 ℃ overnight, and subsequently with biotinylated anti-mouse secondary antibody, followed by alkaline phosphatase–streptavidin at 37 ℃ for 40 min. For visualization of immunostaining, a DAB kit (ZSGB-BIO) was used and the slices were counterstained with hematoxylin.

Constitution and Storage of the Panel

In order to establish the PrP^{Sc} panel of brain tissues from experimental hamsters, 30 brain tissue samples from hamsters infected with scrapie strain 263K and 30 brain tissue samples from normal hamsters were enrolled. The presence of PrP^{Sc} in each brain tissue sample was confirmed with PrP-specific Western blot and partially with immunohistochemical assay. The panel was divided into PrP^{Sc} positive group and PrP^{Sc} negative group, according to the presence or absence of PrP^{Sc} signals. The constitution of the panel is summarized in Table 1. Briefly, PrP^{Sc} positive group consisted of 30 preparations of 10% (signed as S1), 1% (signed as S2) and 0.5% (signed as S3) brain homogenates from 30 scrapie infected hamsters, respectively. Similarly, PrP^{Sc} negative group consisted of 30 preparations of 10% (signed as C1), 1% (signed as C2) and 0.5% (signed as C3) brain homogenates from 30 normal hamsters, respectively. All stocks were stored at –80 ℃ till use.

TABLE 1

Constitution of a Prion Disease PrP^{Sc} Panel of Brain Tissue Samples from Normal and Experimental Hamsters Infected with Scrapie Agent 263K

Groups	Animals (*n*)	Brain Homogenate (% w/v)	Serial Number	Aliquots per Sample	Total
PrP^{Sc} Positive	30	10 %	S1	17.3	523
		1 %	S2	15.3	460
		0.5 %	S3	20	600
PrP^{Sc} Negative	30	10 %	C1	15	450
		1 %	C2	15	450
		0.5 %	C3	15	450

RESULTS

Detection of PrP^{Sc} in the Brain Tissue Samples from Experimental Hamsters

Thirty hamsters were intracerebrally inoculated with scrapie strain 263K as previously described. After a similar incubation period (66.7 ±1.1 d), all tested hamsters demonstrated typically clinical manifestations of prion diseases[10]. The animals were killed at the ultimate stage of the disease and 10% homogenates of brain tissues were prepared. As expected, Western blot assay with PK digestion revealed the PrP^{Sc}-specific bands mobilizing at the positions from *Mr* 21 to 27 KD in all brain homogenates from infected hamsters (Fig. 1A), while no positive signals in any brain homogenate from normal hamsters were present (data not shown). Immunohistochemical assays for parts of infected animals identified widely distributing deposits of PrP^{Sc} signals in various brain regions, mostly tiny spots (Fig. 1B), confirming the wide distribution of PrP^{Sc} in brain tissues of infected hamsters.

Evaluation of Amount of PrP^{Sc} in Different Stocks of Preparations in the Panel

To get detailed information about PrP^{Sc} in different stocks in the panel, all stocks of 1% and 0.5% brain homogenates obtained by 1:10 and 1:20 dilution from 30 PrP^{Sc}-positive 10% brain homogenates from infected hamsters were further analyzed by Western blot. PrP^{Sc} signals were observed in 86.7% stocks of 1% homogenates (26/30 stocks) and 63.3% stocks of 0.5% homogenates (19/30 stocks, Table 2) in a concentration-dependant manner, indicating that the amount of PrP^{Sc} in brain tissues of scrapie 263K-infected hamsters vary each other in the ultimate clinical stages, although their incubation periods of time were quite similar.

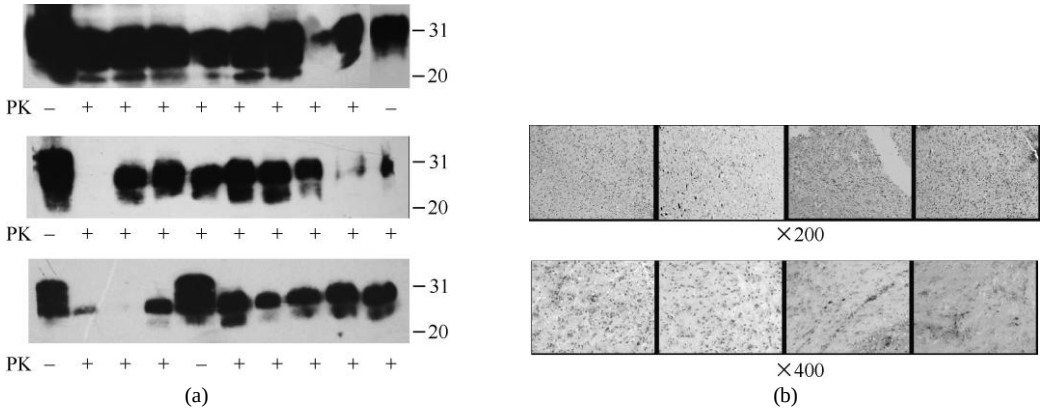

FIG. 1. Detection of PrP^{Sc} in the brain tissue samples from normal (A) by Western blot and scrapie strain 263K-infected hamsters (B) by immunohistochemistry. Ten percent brain homogenates of normal or scrapie agent 263K infected hamsters were treated with (+) or without (-) 50 μg/mL PK at 37 ℃ for 2 h, then subjected to Western blot assay. The molecular markers are shown on the right.

TABLE 2

Evaluation and Stability Assays for PrP^{Sc} Signals in the Panel

Positive PrP^{Sc} Samples	Origin		3-year Later		Total
	Positive	Negative	Positive	Negative	
10% BH[1] (S1)	30 (100%)	0 (0%)	30 (100%)	0 (0%)	30
1% BH[1] (S2)	26 (86.7%)	4 (13.3%)	25 (83.3%)	5 (16.7%)	30
0.5% BH[1] (S3)	19 (63.3%)	11 (36.7%)	20 (66.7%)	10 (33.3%)	30

Note. [1]BH: Brain Homogenate.

Analysis of Stability of PrP^{Sc} Signals in the Panel

To assess the stability of PrP^{Sc} signals in the panel conserved at -80 ℃, the presence of PrP^{Sc} in each stock was tested by Western blot in half a year and 3 years later, respectively. All the 30 brain tissue samples of 10% brain homogenates were PrP^{Sc} positive after storage for 6 months (data not shown). Three years later, all the 90 stocks of PrP^{Sc} positive samples were reevaluated by Western blotting and strong PrP^{Sc} signals were seen in all stocks of 10% brain homogenates (group S1). Clear PrP^{Sc} signals were also detected in 83.3% of stocks of 1% brain homogenates (25/30) and in 66.7% of stocks of 0.5% brain homogenates (20/30), respectively (Fig. 2, Table 2). Compared with the data obtained when the panel was established, the positive ratios and signal intensities of PrP^{Sc} in all stocks of three-concentration brain homogenates revealed no remarkable change, suggesting that the PrP^{Sc} signals are fairly stable under the storage conditions.

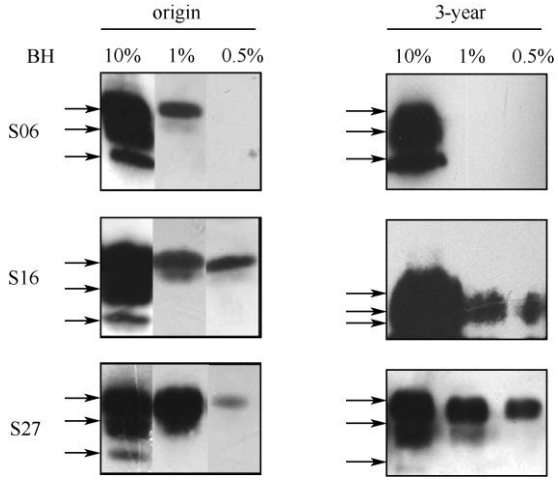

FIG. 2. Stability analysis of the panel after 3 years. Various stocks were kept in a lysis buffer at -80 ℃ for 3 years and PrP signals were detected by Western blot after PK digestion. Arrows on the left indicate three different glycoforms, di-, mono-, and unglycosylated PrP^{Sc}. Dilutions of brain homogenates were shown on the top. BH: brain homogenates; S06, S16, and S27 are the serial numbers. Original and 3-year represent as the preparations when the panel was established and stored for three years later, respectively.

Analysis of Stability of PrP^{Sc} Glycoforms in the Panel

Based on the two potential sites of N-linked glycosylation within PrP protein, three kinds of PrP molecules, di-, mono-, and unglycosylated PrP, were natively observed in brains of both health and TSE animals and humans[11]. To investigate the potential alteration of PrP^{Sc} glycoforms in the panel during the long-time storage, the glycosylating profiles of PrP^{Sc} molecules in the stocks stored at -80 ℃ for three years were analyzed by Western blotting in comparison with the outcomes tested when the panel was established. A retrospective review of the patterns of PrP^{Sc} signals in three different concentrations of the stocks tested when the panel was established revealed that in 10% brain homogenates, 27 out of 30 stocks presented 3 bands with di-, mono-, and unglycosylated PrP^{Sc} molecules, and 3 stocks presented 2 bands with di-, and mono-glycosylated PrP^{Sc} molecules. In 1% and 0.5% brain homogenates, none of the stocks showed three bands,.while 8 and 3 stocks presented 2 bands, and 18 and 16 stocks contained one band, indicating that only di-glycosylatd PrP^{Sc} molecule is contained in the respective stocks (Fig. 2, Table 3), and that the di-glycosylated PrP^{Sc} is the predominant molecule in brains of the hamsters infected with agent 23K, followed by mono-glycosylated and un-glycosylated molecules. In line with the above observations, Western blotting of the stocks stored for three years at -80 ℃ showed a comparable pattern (Fig. 2), that is, in 10% brain homogenates, the number of stocks containing three, two and one PrP^{Sc} bands was 25, 3, and 2 respectively, in 1% brain homogenates (0, 10, and 15) and in 0.5% brain homogenates (0, 3, and 17) (Table 3), indicating that the patterns of glycosylation of PrP^{Sc} molecules in brain homogenates stored under experimental conditions are almost unchanged, revealing a reliable stability of the glycosylation profiles

TABLE 3

Stability of Three PrP^{Sc} Signals in the Panel

Signals	10% BH[1] Stock (S1)		1% BH[1] Stock (S2)		0.5% BH[1] Stock (S3)	
	Origin	3-year	Origin	3-year	Origin	3-year
Three Bands[2]	27 (90.0%)	25 (83.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Two Bands[3]	3 (10.0%)	3 (10.0%)	8 (26.7%)	10 (33.3%)	3 (10.0%)	3 (10.0%)
One Band[4]	0 (0%)	2 (6.7%)	18 (60.0%)	15 (50.0%)	16 (53.3%)	17 (56.7%)
Negative	0 (0%)	0 (0%)	4 (13.3%)	5 (16.7%)	11 (36.7%)	10 (33.3%)
Total	30	30	30	30	30	30

Note. [1]BH: Brain Homogenate. [2]Three Bands: Representing Presences of the Diglycosyl, Monoglycosyl and Aglycosyl PrP^{Sc}. [3]Two Bands: Representing Presences of the Diglycosyl and Monoglycosyl PrP^{Sc}. [4]One Band: Representing Presence of the Diglycosyl PrP^{Sc}.

DISCUSSION

We established a PrP^{Sc} panel of brain tissues from experimental hamsters, which consists of 30 brain tissue samples from hamsters infected with scrapie agent 263K and 30 brain tissue samples from normal hamsters. Each sample contained three stocks with different concentrations, in which the presence of PrP^{Sc} was tested and evaluated. Since the sequences of PrP are highly conserved between different species and different animals have TSE, the similarity in immunoreactivity of prion proteins makes it possible to use the same diagnostic technique for TSE. Therefore, the panel we prepared can be used to evaluate the diagnostic techniques of human and animal prion diseases. A variety of new measurement technologies, including protein misfolding cyclic amplification (PMCA) reported by Saborio and his co-workers in 2001[5], amyloid seeding assay (ASA) developed by Colby and his co-workers in 2007[6], are able to detect traces of PrP^{Sc} in the body fluids. Other techniques, like multi-spectrum UV-fluorescence analysis[7], plasminogen bead assay[8] and confocal dual-color fluorescence correlation spectroscopy[9], are in the process of development, mostly based on the other biochemical characteristics of PrP^{Sc} rather than on immunoreactivity. Although PrP^{Sc} has its molecular features, such as glycosylation profile and electrophorestic pattern, its clinical features and pathological features in various brain regions, vary among different TSEs. The high similarities of PrP^{Sc} in biology and biochemistry make these techniques be used in treatment of TSE. The panel reported in this study provides an opportunity for evaluating the diagnostic techniques based on non-immunological characteristics of PrP.

In this PrP^{Sc} panel, all brain homogenates from infected and normal hamsters were grouped into three concentrations, 10%, 1%, and 0.5%. Information about the PrP^{Sc} signal in each stock,was carefully analyzed and linked into the database of the panel. As we expected, in part of 1% and 0.5% brain tissue stocks, no PrP^{Sc} signal was detectable by Western blot, reflecting a detecting limit or grey zone of this specific assay. As a widely used method, Western blot has been recommended by WHO and OIE to ascertain the diagnosis of human and animal prion disease[12]. Since Western blot has a reliable specificity and a relatively good sensitivity, the results of PrP^{Sc} tested by Western blot have been accepted as one of the golden criteria. Therefore, the PrP^{Sc} panel can be used as an evaluating standard for the sensitivity of a new diagnostic technology. In addition, the brain tissue samples from normal hamsters could also provide a criterion for evaluating the specificity.

Our data confirm that PrP^{Sc} immunoreactivity, PK-resistance and glycosylation profile in brain homogenates did not change much after three years storage. Infectivity of prion is incredibly stable when exposed to convenient physical and chemical agents. As a unique indicator, PK-resistance is used to distinguish PrP^{Sc} from PrP^{C} *in vitro*, although our previous data reveal that the PK-resistant activity of PrP^{Sc} *in vitro* cannot fully represent its infectivity *in vivo* and the stability of PK-resistance of PrP^{Sc} is weaker than its infectivity when exposed to physical and chemical factors[13]. The presence of PrP^{Sc} molecules in the stocks, indicated as its PK-resistance, confirmed a long-term maintenance of PrP^{Sc} biochemical characteristics under the storage conditions. Moreover, glycoforms of PrP^{Sc} in various stocks almost remained unchanged after storage for 3 years. The diversity in glycosylation leads to a variety of glycosylated forms of PrP^{Sc}[14]. Moreover, PrP glycoform and the relative mobility of unglycosylated PrP^{Sc} are increasingly used as a method to distinguish different TSE strains[15-16]. In

line with the observation of PrP^{Sc} molecules in brains of 263K-infected hamsters, the diglycosylated form of PrP^{Sc} remained predominant after storage for 3 years. Stability of PK-resistance and glycoform of PrP^{Sc} stored in a lysis buffer highlights the feasibility to ensure the quality of the panel for a long time.

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