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Sensitive detection of scrapie prion protein in soil

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ABSTRACT

Prion diseases are fatal neurodegenerative disorders that are caused by infectious agents known as prions. Prions are composed primarily of the pathogenic prion protein isoform, PrP^{Sc}. Because significant levels of infectivity have been detected in excrement from animals infected with scrapie and chronic wasting disease, studies on the dynamics of PrP^{Sc} levels in contaminated soil are needed to assess the possible horizontal transmission of prion diseases. Using protein misfolding cyclic amplification, we developed a sensitive detection method for scrapie PrP^{Sc} that is mixed with soil. Our detection method has the advantage of not requiring extraction of PrP^{Sc} from soil and could provide a sensitivity 1000 to 10,000 times higher than that obtained with an extraction-based method. In addition, we found that PrP^{Sc} levels in experimentally contaminated agricultural soils declined to different extents over the course of a 6-month incubation period. Our method appears to be a very useful technique for monitoring PrP^{Sc} levels in soil.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders that have been described in sheep and goats as scrapie, in cattle as bovine spongiform encephalopathy (BSE), in deer and elk as chronic wasting disease (CWD), and in humans as Creutzfeldt–Jakob disease (CJD) [1]. Prions are the infectious agents responsible for TSEs, which are characterized by the accumulation of the pathogenic scrapie isoform of prion protein (PrP^{Sc}) in the nerve tissues of infected subjects. PrP^{Sc} is protease resistant and has a substantially different conformation than the cellular form of prion protein (PrP^{C}) [2].

Scrapie and CWD are the prion diseases that are known to spread horizontally [3–5]. Therefore, it is suspected that environmental reservoirs, at least in part, mediate the transmission of these diseases. Scrapie prions in soil retain their infectivity for several years [6,7], and this infectivity is heightened when the prions adhere to the mineral components of the soil [8]. In addition, significant levels of infectivity have been detected in the saliva [9], urine [10], and feces [11] of TSE-infected animals. These findings suggest the possibility that some prion diseases spread via contaminated soil.

In the case of BSE, it seems that the cause of the outbreak was feeding cattle PrP^{Sc}-contaminated meat and bone meal (MBM) acquired from rendering carcasses of BSE- or scrapie-infected ruminants [12,13]. Because MBM was also used as a fertilizer, prions might have accumulated for years in farm land on which contaminated MBM was used. Therefore, a sensitive method for assessing PrP^{Sc} levels in soil is needed in order to prevent horizontal transmission of prion diseases. However, the extraction [14] and immunodetection methods [15] reported previously are not very effective at identifying small quantities of PrP^{Sc} in soil.

Protein misfolding cyclic amplification (PMCA) has been shown to be very effective at detecting minute amounts of scrapie PrP^{Sc} [16]. Although PMCA was capable of amplifying PrP^{Sc} extracted from soil [7], a detailed comparison of the sensitivity of the extraction method and PMCA has not been made. In the present study, we report a simple, and highly sensitive method for detecting a mouse-adapted scrapie PrP^{Sc} that is mixed with soil. Using both the extraction method and PMCA, we examined the dynamics of PrP^{Sc} levels in two agricultural soils over the course of a 6-month incubation period.

2. Materials and methods

2.1. Soils

In the present study, two types of agricultural soils were examined: Low-humic Andosols (LHA), which is a volcanic ash soil

Abbreviations: GLS, Gray Lowland soils; LHA, Low-humic Andosols.

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widely distributed in Japan and contains large amounts of organic matter and Gray Lowland soil (GLS), which is a poorly-drained alluvial soil that accounts for a third of the rice paddy fields in Japan. Soil samples were air-dried, sieved through a 2-mm mesh screen, and stored at room temperature (RT).

2.2. Brain homogenates

The mouse-adapted scrapie prion strain Chandler was propagated in ICR mice. The brains of mice at the terminal stage of disease were pooled and homogenized at concentration of 10% (w/v) in PBS. The supernatant was separated by brief centrifugation and used as the PrP^{Sc} solution. The infectivity titer of the brains was calculated by bioassay to be $5 \times 10^{8.25}$ LD_{50} per gram [17].

Brains of normal ICR mice were homogenized at a concentration of 20% (w/v) in PBS containing complete protease inhibitors (Roche Diagnostics). The homogenates were stored in small aliquots at −80 °C until further use. For use, the homogenates were mixed with an equal volume of elution buffer (PBS containing 2% Triton X-100 and 8 mM EDTA) and incubated at 4 °C for 1 h with continuous agitation. After centrifugation at 4500*g* for 5 min, the supernatant was used as the PrP^{C} solution.

2.3. PrP^{Sc} extraction from soil

Because soil adsorbs PrP^{Sc} immediately and firmly, it is difficult to extract PrP^{Sc} from some types of soil even in the presence of a detergent such as SDS [14]. Methods for PrP^{Sc} extraction from soil have been reported previously [18,19], and we modified the extraction procedure to obtain maximum recovery of PrP^{Sc} from soil as follows: Serial 10-fold dilutions of the infected brain homogenate were prepared in 1% normal mouse brain homogenate. Air-dried soils (GLS and LHA, 100 mg each) were mixed with 33 μl of the diluted infected brain homogenates (0.001–1% for GLS and 0.001–10% for LHA) and incubated at RT for 1 h. The soil samples were mixed with 100 μl of proteinase K (PK, 200 μg/ml) in 100 mM sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 1 h with agitation. To inhibit the PK digestion, 8.3 μl of 100 mM phenylmethylsulfonyl fluoride (PMSF) in methanol was added to the samples and incubated with agitation at RT for 10 min. To release PrP^{Sc} from soils effectively, 10 μl of 20% SDS in water and 10 μl of 100 mM EDTA in PBS were added to the samples and incubated with agitation at RT for 5 min. After centrifugation at 4500*g* for 5 min, supernatants were collected. The supernatants were mixed with equal volumes of 2× SDS sample buffer and incubated at 100 °C for 5 min. PrP^{Sc} signal was detected by western blotting (WB) as described below.

2.4. PrP^{Sc} incubation in soils

To determine the optimal amplification conditions for PrP^{Sc} in soil, a preliminary experiment was performed using LHA soil. Air-dried and ground soil samples (8 mg) were mixed with 5 μl of the serially diluted infected brain homogenates (1–0.001%). After incubation at RT for 10 min, 100 μl of blocking reagent (Blocking One, Nacalai Tesque) was added to the PrP^{Sc}-soil mixture and incubated for 30 min with agitation to block any protein-binding sites of the soil particles. The soil suspensions were used directly as seeds for PMCA analysis.

In the present study, the changes of PrP^{Sc} level in two types of soil, GLS and LHA, were examined during an incubation period of 6 months. Air-dried soil samples (100 mg) were mixed with 33 μl of the serially diluted infected brain homogenates (0.001–1% for GLS and 0.001–10% for LHA) and kept in an incubator at 25 °C. The soil samples were examined immediately after preparation and after 1, 3, and 6 months of incubation. The samples were suspended in blocking reagent at a concentration of 8 mg of soil/ml and incubated for 30 min with agitation. The soil suspensions were used directly as seeds for PMCA analysis.

2.5. PMCA

We previously demonstrated that PrP^{Sc} derived from the Chandler strain could be amplified *in vitro* to infinite levels in the presence of digitonin [17]. We found that Chandler PrP^{Sc} could be amplified efficiently in the absence of digitonin when pulse oscillation was repeated intermittently with a short interval. In the present study, PMCA was performed using a fully automatic cross-ultrasonic protein activating apparatus (Elestein 070-GOT, Elekon Science Corporation), with 60 cycles of sonication in which a 3-s pulse oscillation was repeated five times at 0.1-s intervals followed by incubation at 37 °C for 30 min with gentle agitation. For examination of the sensitivity of this method for the detection of Chandler PrP^{Sc}, the 10% infected brain homogenate was serially diluted from 10^{-4} to 10^{-10} with the PrP^{C} substrate (total volume, 100 μl) in an electron-beam irradiated polystyrene tube. The amplified product in the first round of amplification was diluted 1:10 with the PrP^{C} substrate, and a second round of amplification was performed. The process was repeated to obtain the third-round amplification products.

2.6. Western blotting

For the PMCA products, samples (10 μl) were mixed with 10 μl of PK solution (100 μg/ml) and incubated at 37 °C for 1 h. The digested materials were mixed with 20 μl of 2× SDS sample buffer and incubated at 100 °C for 5 min. The samples were separated by SDS–PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Bradford, MA). After blocking, the membrane was incubated for 1 h with horseradish peroxidase-conjugated monoclonal antibody T2 [20] at a 1:10,000 dilution. After washing, the blotted membrane was developed with Immobilon Western Chemiluminescent HRP substrate (Millipore) according to the manufacturer's instructions. Chemiluminescence signals were analyzed using the Light Capture system (ATTO, Tokyo, Japan).

3. Results

3.1. Detection sensitivity of PMCA for PrP^{Sc}

We first examined the detection sensitivity of the amplification method for the Chandler PrP^{Sc}. Fig. 1 shows the results from the amplification of each diluted sample. Before amplification, the PrP^{Sc} signal was detected in the samples diluted up to 10^{-3} (data not shown). After the first round of the PMCA reaction, PrP^{Sc} signals could be detected in the samples diluted up to 10^{-7}, corresponding to 1 ng of infected brain in 100 μl of the PrP^{C} substrate, but the signal intensities were quite weak. The PrP^{Sc} signals were enhanced in the second round of amplification. After the third round of amplification, PrP^{Sc} signals were clearly detected in the samples diluted to 10^{-8} and 10^{-9}. No signal was detected for the more extreme dilutions even after four rounds of amplification. The eight control samples containing only PrP^{C} substrate did not show any signal in the sequential PMCA analysis.

3.2. Amplification of PrP^{Sc} in soil

Fig. 2 illustrates the results of the amplification of PrP^{Sc} adsorbed in the LHA soil samples. The PrP^{Sc}-soil suspension in 100 μl of the blocking reagent contained 8 mg of soil and 50 ng–50 μg of infected brain. When 8 μg of soil (equivalent to 0.1 μl of each PrP^{Sc}-soil suspension) was used as the PMCA seed, a signal with three bands corresponding to the three glycosylated forms of PrP^{Sc} was detected in both samples containing 50 ng of infected brain and in one of the two