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Capillary electrophoresis of the scrapie prion protein from sheep brain

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Abstract

Scrapie in sheep and goats causes a progressive, degenerative disease of the central nervous system and is the prototype of other transmissible spongiform encephalopathies (TSE) found in humans and in animals. In samples of TSE-affected brains, unique rod-shaped structures are found and are infectious. These rods are composed of a protease-resistant, post-translationally modified cellular protein (PrP^{sc}) that has a molecular mass of ca. 27 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Laboratory tests used for the diagnosis of scrapie detect PrP^{sc}. The overall concentration of PrP^{sc} in tissues is low. The present methods to diagnose scrapie are lengthy, require relatively large quantities of starting material to detect PrP^{sc} and lack sensitivity. We explored the use of free zone capillary electrophoresis and immunocomplex formation to detect PrP^{sc} in the brain tissue of infected sheep. Brain tissue from both infected (as confirmed by histological and biological tests) and from normal animals was used to prepare the PrP^{sc}. After treatment with proteinase K and non-ionic detergents, PrP^{sc} was solubilized and reacted with a rabbit antiserum specific for a peptide of the prion protein. Immunocomplex formation was observed for the samples from scrapie-infected brain but not for samples from normal brain. When a fluorescein-labeled goat anti-rabbit immunoglobulin was used as a second antibody, the detection of immunocomplex formation was enhanced both by the immunological technique and by using laser-induced fluorescence for detection. This same rabbit antiserum was used on immunoblot analysis. Three bands were observed for material from an infected sheep but none in preparations from brain material from normal sheep. Capillary electrophoresis can be used to show immunocomplex formation when PrP^{sc} is present in sheep brain.

1. Introduction

Scrapie in sheep and goats causes a progressive, degenerative disease of the central nervous system. Scrapie is the prototype of other transmissible spongiform encephalopathies (TSE) found in humans and in animals [1]. In animals affected with TSE, unique rod-shaped structures called prion rods or scrapie-associated fibrils (SAFs) accumulate in the central nervous system of affected individuals [2]. In the course of infection, a normal cellular glycoprotein isoform (PrP^{c}) [3] is truncated at the N terminus by a lysosomal protease(s) [4] and acquires the ability to form SAFs that are protease resistant. The pathogenesis and molecular basis of how these SAFs cause nerve cell loss is not understood but may be related to chronic exposure to the SAFs and their degradation products [5]. Mice, that have had the normal cellular gene for PrP^{c}

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altered, were unable to express this protein and were resistant to infection with the scrapie agent [6]. When SAFs are extracted from brain of scrapie-infected animals, both detergent extraction and treatment with proteinase K are necessary for the formation of scrapie prion rods in vitro [7]. Treatment of SAFs with 2-β-mercaptoethanol and sodium dodecyl sulfate (SDS) dissociates the SAFs into a protein of a molecular mass of ca. 27 000 on SDS-polyacrylamide gel electrophoresis (PAGE). This monomer is called the prion protein (PrP^{sc}). Although treatment with detergent and digestion with proteinase K produces a highly enriched fraction of PrP^{sc}, proteins, peptides and fatty acids are present [8]. The concentration of PrP^{sc} in the tissues of infected animals is low. In early stages of the disease, PrP^{sc} is associated with the follicular dendritic cells of the spleen and lymph nodes [9,10]. Present methods that are used to detect PrP^{sc} are lengthy, require relatively large quantities of starting material, and lack sensitivity. The small amounts of material required for capillary electrophoresis (CE) coupled with the ability to do several kinds of protein analyses under conditions that do not destroy the normal functions of the proteins suggest that this may be an excellent technique to detect the presence of PrP^{sc} [11–15]. In this paper we describe conditions in which free zone CE using both UV and laser-induced fluorescence detection was used to identify immunocomplex formation of PrP^{sc} with a rabbit antiserum specific for a peptide of PrP^{sc} [16].

2. Materials and methods

2.1. Preparation of the prion protein

Brain stem samples that were from confirmed cases of scrapie by histopathological examination and by immunohistochemistry and from normal sheep were used. A 1-g amount of brain stem was homogenized with a Brinkman Polytron (Brinkman Instruments, Westbury, NY, USA) for 60 s in 20 ml of 10% (w/v) sucrose in a 40-ml centrifuge tube. After homogenization the suspension was centrifuged at 10 000 *g* for 30 min. The supernatant fraction was saved and the pellet was washed with 20 ml of 0.01 *M* Tris pH 7.4 containing 0.15 *M* NaCl and centrifuged as above. The two supernatant fractions were pooled and centrifuged at 230 000 *g* for 45 min. The pellet was resuspended in 0.01 *M* Tris pH 7.4 containing 0.15 *M* NaCl and centrifuged at 230 000 *g* for 45 min. The pellet was held on ice for 4°C overnight and then resuspended in 10 ml of 0.01 *M* Tris pH 7.4 containing 5% sarcosyl and incubated for 0.5 h at room temperature. After incubation, the sample was centrifuged at 10 000 *g* for 15 min. The supernatant fraction was centrifuged at 230 000 *g* for 1.5 h. The pellet was resuspended in 1 ml of 0.01 *M* Tris pH 7.4 and incubated with 20 μg proteinase K at 37° for 30 min. The reaction was stopped with 50 μl of 0.006 *M* phenylmethylsulfonyl fluoride. The sample volume was increased to 20 ml with 0.01 *M* Tris pH 7.4 and centrifuged at 230 000 *g* for 1.5 h. The pellet was resuspended in 100 μl of 0.01 *M* Tris pH 7.4 and stored at −70°C. Samples from scrapie-infected sheep brain were designated SBT and those from normal sheep brain were designated NBT.

2.2. SDS-PAGE and immunoblots

Samples from above were diluted 1:2 in 0.24 *M* Tris·HCl pH 6.6 that contained 2% SDS. The samples were made 2% (v/v) in β-mercaptoethanol and boiled for 10 min. The samples were applied to two pre-formed 10–15% gradients gels on the Phast Gel apparatus (Pharmacia LKB, Uppsala, Sweden) according to the directions of the manufacturer. After SDS-PAGE, one of the gels was stained with a Rapid Coomassie Stain (Research Products, Mt. Pleasant, IL, USA) according to the directions of the manufacturer. This stain has a sensitivity of detection of 8 ng of protein. The samples from the other gel were transferred to nitrocellulose paper using diffusion at 70°C for 20 min on the Phast Gel apparatus. The method used to develop the immunoblot was a modification of Hawkes et al. [17]. The blot was blocked with 0.05 *M* Tris pH 7.5 + 0.2 *M* NaCl (Tris-buffered

saline, TBS) containing 1.5% fish gelatin (Sigma, St. Louis, MO, USA) for 1 h. After three 10-min washes with TBS containing 0.15% fish gelatin, a dilution of rabbit serum 27 (antiserum made to the peptide 89–103 of the PrP^{sc} [4,16]) of 1:1000 was placed on the blot and left overnight at 25°C. The blot was then washed as above and a dilution of 1:1000 of biotinylated protein G (Zymed Labs., San Francisco, CA, USA) was added and incubated for 1 h and then washed as above. A 1:1000 dilution of streptavidin labeled with horse radish peroxidase (Pierce, Rockford, IL, USA) was added and incubated for 1 h and the blot was then washed as above. Using the procedure of the manufacturer, the color reagent 1-chloro-4-naphthol (Bio-Rad Labs., Richmond, CA, USA) was added and the bands allowed to develop for 45 min.

2.3. Free zone capillary electrophoresis

CE was performed on a P/ACE system Model 2050 (Beckman Instruments, Fullerton, CA, USA) controlled by System Gold software (Beckman). UV absorbance was monitored by a fixed-wavelength detector at 214 nm. Laser-induced fluorescence (LIF) detection was done using an air-cooled Ar ion laser (Beckman), excitation 488 nm and emission 560 nm. Capillaries were obtained from Beckman. The capillary was 57 cm (50 cm to the detector) × 75 μm I.D. The separating voltage was 15 kV using 0.1 *M* sodium borate pH 8.5. Before the samples were injected, the capillary was rinsed with 0.1 *M* NaOH for 1 min followed by a 2-min rinse with distilled water. The capillary was then equilibrated with borate buffer by rinsing for 4 min. Samples were injected for 10 s. The samples included: (1) rabbit antiserum 27, (2) SBT, (3) NBT, (4) SBT + rabbit antiserum and (5) NBT + rabbit antiserum. Samples for LIF detection included: (1) fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulins (FITC-G × RbIg), (2) FITC-G × RbIg + rabbit antiserum, (3) FITC-G × RbIg + rabbit antiserum + SBT and (4) FITC-G × RbIg + rabbit antiserum + NBT.

2.4. SDS-Gel capillary electrophoresis

eCAP SDS 200 and eCAP SDS 14-200 kits were purchased from Beckman. The same CE system as described above was used. The manufacturer's directions were followed to perform the electrophoresis. Standards (Beckman) consisting of proteins of known molecular masses were run. The samples, SBT and NBT were prepared for these capillaries in the same manner as for SDS-PAGE.

2.5. Preparation of immunocomplexes

Immunocomplexes were prepared by incubating dilutions of the rabbit antiserum with SBT and NBT and the FITC-G × RbIg. Dilutions of both the rabbit antiserum and the FITC-G × RbIg were made in 0.05 *M* sodium phosphate pH 7.2 containing 0.15 *M* NaCl (phosphate-buffered saline, PBS). The fluorescein-labeled goat anti-rabbit immunoglobulins (Zymed Labs.) were purified over a fast flow protein G column (Pharmacia LKB) using the directions of the manufacturer for the chromatography. Fluorescence from the column fractions was monitored with the P/ACE system by rinsing the column fraction into the capillary and reading the fluorescence at 1 min. Peak fractions that were eluted from the protein G column were pooled and the pH adjusted to 7.0 by the addition of PBS. The pool was distributed into 50-μl aliquots and stored at −70°C. The dilution of the FITC goat anti-rabbit immunoglobulins used was 1:200. Lower dilutions had an increased noise-to-signal ratio. The final dilution of the specific rabbit antiserum was used at a 1:20. The SBT or NBT were not diluted.

3. Results

The results of the Coomassie Blue staining of SDS-PAGE of the protease-treated infected and normal sheep brain samples are shown in Fig. 1A. In order to obtain detectable bands ca. 5 μl of the preparations of SBT or NBT were required. In order to clearly observe these bands

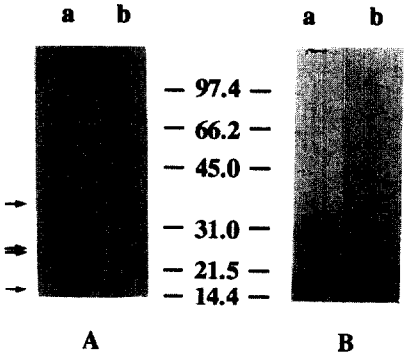

Fig. 1. SDS-PAGE and immunoblots of preparations from scrapie-infected sheep brain and from normal sheep brain. (A) SDS-PAGE, Coomassie Blue stain; lanes: a = scrapie-infected brain; b = normal brain; light bands are indicated by arrows. (B) Immunoblot; lanes as in (A). Molecular mass markers are indicated by arrows; molecular masses $\times 10^{-3}$.

ca. 10 times more sample is needed. Both the normal and infected sheep brain have a faint protein band at the same molecular mass of ca. 35 000. The infected sheep brain had additional bands that stained at M_r 25 000, 23 000 and 15 000. Upon immunoblot analysis (Fig. 1B), no bands were observed for the preparation from normal sheep brain. For the infected sheep brain, three bands that were more diffuse than the bands staining with Coomassie Blue were observed at M_r 29 000, 25 000 and 19 000. To detect PrP^{sc} on immunoblot at least 5 μl of the sample were required.

Electropherograms were produced using the same material that was prepared for SDS-PAGE. A representative electropherogram from an eCAP SDS 14-200 capillary is shown in Fig. 2.

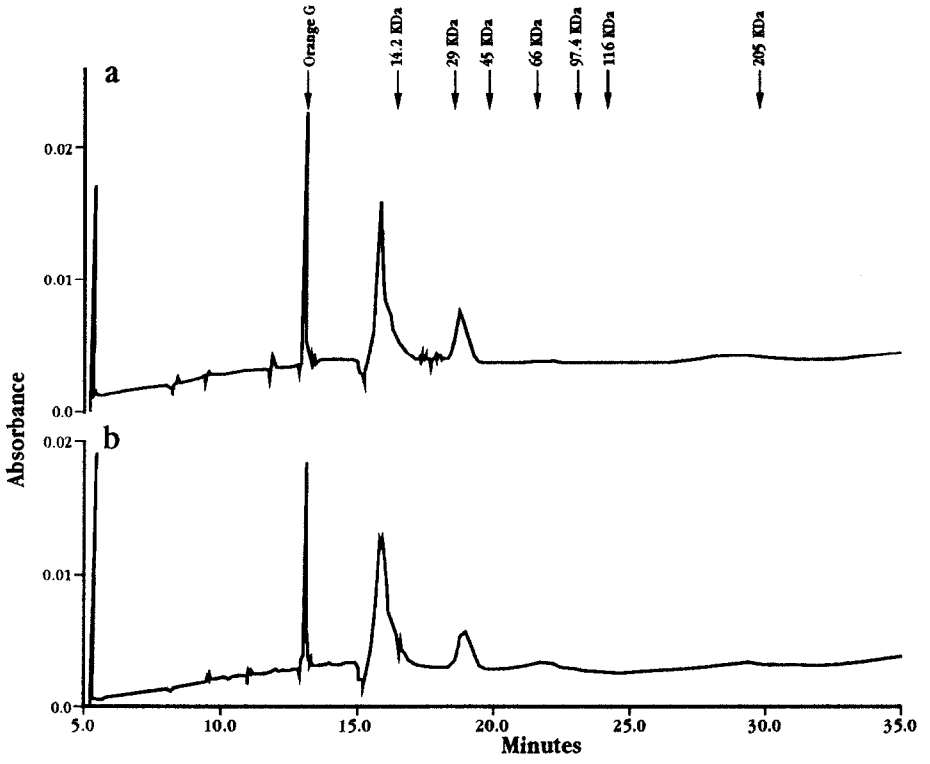

Fig. 2. eCAP SDS 14-200 Electropherograms of sheep-brain preparations. Arrows indicate where proteins of known molecular mass (KDa = kilodalton) migrate. (a) Proteinase K-treated scrapie-infected sheep brain; (b) proteinase K-treated normal sheep brain.

Approximately, 30 nl were used in this injection. From this analysis, two proteins were observed for both the normal and infected sheep brain preparations. One was at M_r 29 000 and 12 000. When ca. 175 times more sample was used for SDS-PAGE, barely visible bands were observed using Coomassie staining and immunoblot analysis. Normal brain showed only one faint band and as can be seen in Fig. 2 two peaks clearly are observed. A protein at M_r 29 000 was obtained from an eCAP SDS200 but the protein at $M_r \approx$ 12 000 was not resolved on the eCAP SDS200 (data not shown).

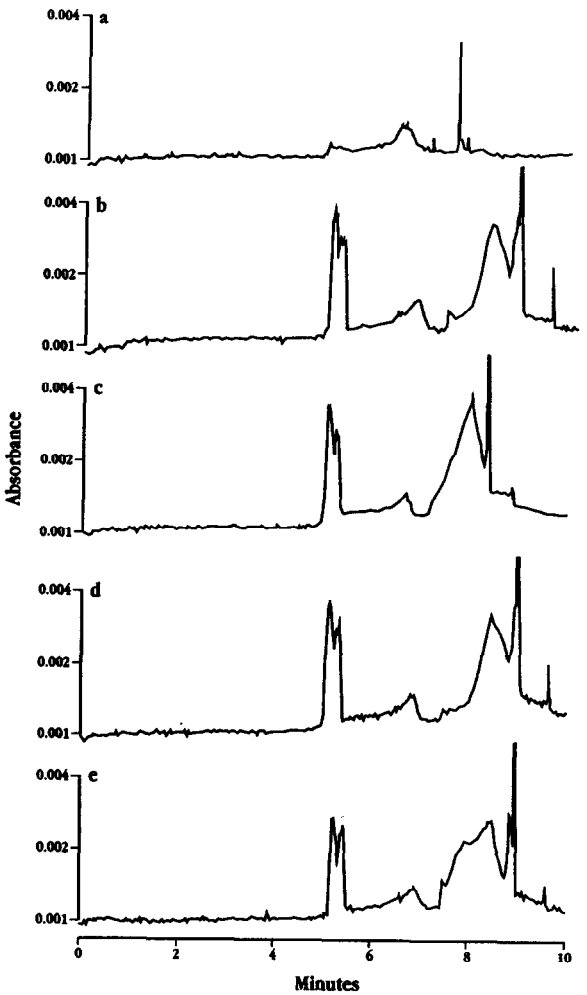

Fig. 3. Free zone CE using UV absorbance for detection of immunocomplex formation. (a) Rabbit antiserum 27 diluted 1:20; (b) proteinase K-treated scrapie-infected sheep brain; (c) proteinase K-treated normal sheep brain; (d) rabbit antiserum 27 1:20 + proteinase K-treated normal sheep brain; (e) Rabbit antiserum 27 1:20 + proteinase K-treated scrapie-infected sheep brain.

Representative electropherograms that were obtained from free zone CE using UV detection are shown in Fig. 3. Ca. 30 nl of the brain preparations were injected. The dilution of rabbit antiserum that showed immunocomplex formation was 1:20. Immunocomplex formation is shown in Fig. 3e (SBT + rabbit antiserum) in which an additional peak is observed on the electropherogram at approximately 8.0 min. This does not appear on the normal brain sample (Fig. 3d, NBT + rabbit antiserum).

Representative electropherograms that were obtained using LIF for detection are shown in Fig. 4. A significant difference (peak at ca. 7.5 min) is shown between the mixture containing NBT (Fig. 4c) vs. that containing SBT (Fig. 4d).

4. Discussion

The diagnosis of scrapie in sheep is generally dependent on observation of clinical signs and subsequent identification of lesions. Recently, other tests using immunohistochemistry and immunoblot techniques have been used to identify the PrP^{sc} whose presence is characteristic of this disease. The outbreak of a similar disease in cattle (bovine spongiform encephalopathy) pointed to the need of a more rapid and a more sensitive test to diagnose the disease before the onset of clinical symptoms. CE was explored as a possible means to identify PrP^{sc} in samples from potentially infected animals and to aid in pathogenesis studies of the disease.

In this study, there was general agreement between SDS-PAGE and CE on the molecular masses observed. The presence of small molecular mass discrepancies ($\pm$ 2000) were noted for the SDS-PAGE and the CE measurements. The fact that this protein is a glycoprotein could contribute to these slight differences in the molecular masses. These molecular masses are within the range reported in the literature [10]. Based on volume, the amount of material

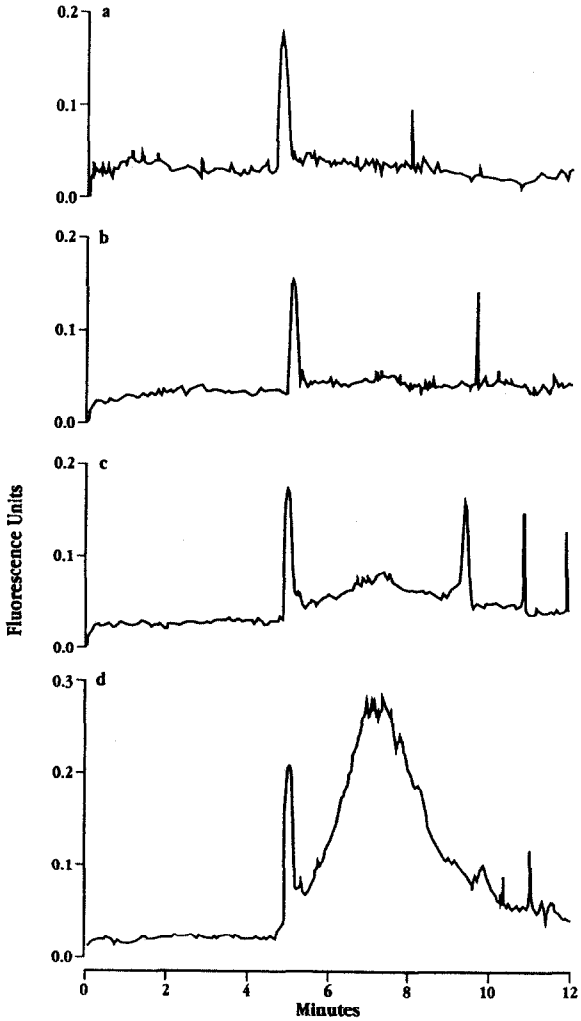

Fig. 4. Free zone CE using LIF detection of immunocomplexes. (a) FITC-G × RbIg; (b) FITC-G × RbIg + rabbit antiserum 1:10; (c) FITC-G × RbIg + rabbit antiserum 1:10 + proteinase K-treated normal sheep brain; (d) FITC-G × RbIg + rabbit antiserum 1:10 + proteinase K-treated scrapie-infected sheep brain.

needed to perform the CE analysis was ca. 200 times less than the amount needed for the SDS-PAGE.

When free zone CE was performed on the samples, it was only in the presence of rabbit antiserum, which formed immunocomplexes, that differences were observed between the NBT and SBT preparations. These electropherograms are more complex to interpret than those reported in the literature [18] in which highly purified proteins and monoclonal antibodies were used. The samples in this study were not as highly purified in that the rabbit antiserum contained polyclonal antibody and that the brain preparations contained more than one protein. Under these conditions, the immunocomplexes would be expected to be more heterogeneous than those prepared from highly purified proteins and Fab fragments of monoclonal antibodies. For these reasons, it was important to include the NBT as a control in these experiments. Immunocomplex formation was observed using UV detection for the primary reaction between antibody and the SBT. This was reproducible using different brain preparations and different separating voltages. Schroll et al. [19] reported that the ratio of antigen (SBT) to antibody was important to immunocomplex formation. When we used two different dilutions of the rabbit serum, the dilution of 1:20 produced the most immunocomplexes. Dilutions of the antigen (SBT) were not done in this study primarily because of the low concentration of PrP^{sc} in the preparations. The use of a fluorescein-labeled second antibody to the rabbit antibody enhanced the detection of the immunocomplexes. Although some immunocomplex was observed with unpurified FITC-G × RbIg (data not shown), affinity purification of FITC-G × RbIg reduced the complexity of the electropherogram and made it easier to observe the immunocomplexes. Because capillary electrophoresis requires minute amounts (nl quantities) of material and because of the sensitivity of detection, we were successful in distinguishing between normal and scrapie-infected sheep brain.

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