Western Blot Detection of Scrapie-associated Fibril Protein in Tissues outside the Central Nervous System from Preclinical Scrapie-infected Mice

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SUMMARY

We describe a method of sample preparation to detect scrapie-associated fibril (SAF) proteins in small amounts of scrapie-infected mouse tissues by Western blot analysis using an antiserum to a synthetic peptide that corresponds to the N-terminal region of hamster prion protein. SAF proteins were efficiently detected in brain tissue by this procedure. The proteins were also detected in preparations from spleen and lymph node. SAF proteins were detected in brain samples at 24 weeks after intraperitoneal infection. Using spleen samples, the proteins were detected from mice in the preclinical stage (from 4 weeks after infection), clinical symptoms of scrapie were observed in some mice from 22 weeks after infection.

Scrapie is a spongiform encephalopathy of sheep and goats. The scrapie agent has been transmitted experimentally between its natural hosts and from the natural hosts to experimental animals such as mice and hamsters (Dickinson, 1976). Infected animals do not develop detectable immune responses to the scrapie agent (Kasper et al., 1981; Kingsbury et al., 1981) and therefore there is still no way of identifying infected animals before the clinical stage.

Unique fibrillary structures of 4 to 6 nm width and 50 to 500 nm length designated scrapie-associated fibrils (SAF) (Merz et al., 1981) or prion rods (Prusiner et al., 1982) were found in fractions from brains of scrapie-infected animals. The concentration of SAF was directly proportional to the titre of infectivity; that is SAF co-purified with the scrapie agent (Bolton et al., 1982; Diringer et al., 1983b; McKinley et al., 1983). SAF are partially protease-resistant and contain a glycoprotein (SAF protein) with an apparent Mr of 15 000 to 30 000 (15K to 30K) (Bode et al., 1985; Bolton et al., 1982, 1985; Diringer et al., 1983a; Hilmert & Diringer, 1984). Antibodies to SAF protein have been developed in animals immunized with SAF (Bendheim et al., 1984; Bode et al., 1985; Diringer et al., 1984; Takahashi et al., 1986) and Western blot analyses have revealed that SAF proteins share antigenic determinants with a protein from a membrane fraction prepared from uninfected animals (Cho, 1986; Meyer et al., 1986; Oesch et al., 1985). It is not clear whether SAF are the scrapie agent, a part of the scrapie agent, or a pathological by-product of scrapie infection that co-purifies with the scrapie agent (Basler et al., 1986; Bolton et al., 1984; Chesebro et al., 1985; Oesch et al., 1985). SAF proteins were detected in a protease-treated SAF fraction from brains of scrapie-infected animals (Bolton et al., 1982; Diringer et al., 1983a; Hilmert & Diringer, 1984; Kascak et al., 1985, 1986; McKinley et al., 1983; Takahashi et al., 1986), but were not detected in the brain tissue until the late period of the preclinical stage (Czub et al., 1986b; Rubenstein et al., 1986).

SAF was also detected in a fraction prepared from scrapie-infected mouse spleens (Merz et al., 1983; Rubenstein et al., 1986) by Western blot analysis (WBA) immunologically (Rubenstein et al., 1986; Shinagawa et al., 1986). Conversely, it was reported that SAF proteins were absent from mouse and hamster spleens that contained high titres of scrapie infectivity (Czub et al., 1986a).
Minced tissue (about 140 mg/1.5 ml plastic centrifuge tube).

Add 7 vol. 5 mM-MgCl₂ in TH and an equal weight of sea sand.

Freeze, thaw and vortex or sonicate in a cup-type sonicator.

Add DNase I (40 μg/100 mg tissue) and incubate at 37 °C for 1 h.

Add twice tissue vol. (a) of 25% Sarkosyl in TH and incubate at room temperature for 30 min.

Centrifuge at 15000 r.p.m. (18000 g) for 5 min at room temperature.

Supernatant Precipitate

Add NaCl equal to original tissue weight and incubate at 4 °C for 16 h.

Centrifuge at 18000 r.p.m. (27000 g) at 4 °C for 40 min (b).

Precipitate (18P) Supernatant

Suspend in TH (40 μl/100 mg tissue); sonicate.

Add proteinase K [0.1 μg/100 mg tissue (c)] and incubate at 37 °C for 30 min.

Add 2.5 vol. ethanol and centrifuge at 15000 r.p.m. (18000 g).

Precipitate (ETP) Supernatant

Dissolve in 4% SDS, heat at 100 °C for 10 min and add an equal vol. of 2× sample buffer for electrophoresis.

Fig. 1. Treatment of a tissue sample to obtain a fraction suitable for detecting SAF protein by Western blot analysis. (a) One g of tissue is defined as 1 ml. (b) Ultracentrifugation may yield a higher recovery of SAF protein. (c) The enzyme concentration at which enzyme-treated samples from infected but not uninfected brains reacted with the anti-peptide serum was chosen. TH represents 10 mM-Tris-HCl pH 7.5.

Similar contradictory reports on the presence of SAF protein-specific mRNA in the spleen have been made; Oesch et al. (1985) detected the mRNA whereas Chesebro et al. (1985) did not.

Four-week-old specific pathogen-free Slc/ICR female mice were inoculated with brain homogenates of the Obihiro strain of scrapie either intracerebrally (i.c.; 10%, 20 μl) or intraperitoneally (i.p.; 1%, 200 μl) (Shinagawa et al., 1985). Five-month-old uninfected mice of the same strain were used as controls. The mice were inoculated i.p. with 440 μg/mouse of BCG vaccine (Kyowa Hakko Kogyo, Tokyo, Japan) at 3 and 6 weeks before sacrifice to stimulate lymphatic organs. Western blot analysis of SAF proteins in the samples using an antiserum to a synthetic pentadecapeptide that corresponds to the N-terminal region of the prion protein (anti-peptide serum) was described previously (Shinagawa et al., 1986). Autoradiography using ¹²⁵I-labelled streptavidin (Amersham) was also used after WBA as recommended by the supplier of the reagent.
The procedure for sample preparation outlined in Fig. 1 was developed using the brains of scrapie-infected mice inoculated i.c. The amount of protein in the 18000 r.p.m. (27000 g) precipitate (18P) can be increased by adding NaCl at a final concentration of 5 to 15% to the sodium N-lauroyl sarcosinate (Sarkosyl) tissue extract and by prolonged incubation at 4 °C. In Fig. 2(a), lane 2, five major protein bands (17K, 21K, 24.5K, 27.5K and 31K) and a smear-like band of 47K to 57K are shown representing the blotting of 18P with the anti-peptide serum. After proteinase K (pK) treatment of the ethanol precipitates (ETP) of 18P, only three major bands (17K, 21K and 24.5K) remained (Fig. 2a, lane 1). Weakly stained broad bands representing higher molecular weights resulted from WBA of 18P from uninfected mice; these, however, disappeared after pK treatment (Fig. 2a, lanes 3 and 4). Protein bands observed in the pK-treated ETP sample were indistinguishable from those obtained from the marker SAF fraction prepared by the published method (Takahashi et al., 1986). Although we did not examine infectivities or morphology of SAF in samples prepared by the present method, the proteins detected with the anti-peptide serum were found only in the samples from scrapie-infected mice and the apparent Mr of the proteins corresponded to those of SAF proteins. Therefore, we concluded that the proteins found in our pK-treated samples were SAF proteins.

The efficiency of SAF protein recovery in 18P from the Sarkosyl extract was estimated by centrifuging the 18000 r.p.m. supernatant (18S) at 305000 g for 2 h and analysing the resulting pellet for SAF protein content. As shown in Fig. 2(a), lanes 5 and 6, some SAF proteins remained in the 18S. However, more than half of the SAF proteins were recovered in 18P (Fig. 2a, lanes 1 and 2) and after pK digestion the ratio of protein band intensity between lanes 1 and 5 was about 2:1. Impurities in ETP monitored by silver staining consisted primarily of low molecular weight products of protein degradation (data not shown).

We investigated whether SAF proteins were detectable in spleen tissue, pooled lymph nodes collected from whole bodies and white blood cells from scrapie-infected mice. Since the amount of the protein in spleen tissue was less than that in brain tissue (Shinagawa et al., 1986), we used [125I]streptavidin for detection of SAF protein–antibody complexes by WBA of spleen, lymph node and white blood cell fractions. The sensitivity of SAF protein detection using [125I]streptavidin was at least 10 times higher than that using the peroxidase reaction (data not shown). The SAF fraction used as a marker (Fig. 2b, c, d, e; lanes M) showed, in addition to the three major bands, one band with an Mr of about 50K which was usually very faint or not detected at all by the enzyme reaction as shown in Fig. 2(a), lane M. WBA of 18P from infected mouse spleens resulted in three major bands corresponding to the major SAF protein bands and a broad band of about 50K (Fig. 2b, lane 2). After pK treatment of ETP three bands corresponding to the major SAF protein bands remained, although the 17K band was weaker than the others (Fig. 2b, lane 1). Approximately 45% of the proteins remained in 18S (Fig. 2b, lane 5). The analogous fraction from uninfected mice contained only small amounts of protein that reacted with the antisera and these proteins were removed after pK digestion (Fig. 2b, lanes 3 and 4). Proteins that reacted with the antisera were also detected in samples prepared from 200 mg of lymph nodes. 18P from lymph node nodes of both infected and uninfected mice contained many proteins which reacted with the antisera (Fig. 2c, lanes 2, 4, 6 and 8). In lymph node ETP from scrapie-infected mice, two major bands representing proteins of 21K and 24.5K and a weak smear-like band located above the major bands were observed, the 17K band, however, was almost absent (Fig. 2c, lane 1). In the pK-treated sample of the 18S from infected mice, three bands corresponding to SAF proteins were observed, although the 17K band was weak (Fig. 2c, lane 5). Recovery of proteins from lymph node ETP resulted in a lower yield than from brain and spleen, about 60% of the proteins remained in the lymph node 18S. The lower recovery of SAF proteins by centrifugation at 18000 r.p.m. from spleen and lymph node samples than from brain samples may be due to a lower concentration and less aggregation of SAF proteins in these tissues. In comparison with the brain preparations, 17K, 21K and 24.5K proteins in samples from infected spleens and lymph nodes markedly decreased after pK treatment (compare lanes 1 and 2, 5 and 6 in Fig. 2a, b and c). This suggests that SAF proteins are present in a more pK-sensitive form in these tissues than in the brain. Due to the difficulty of purification of SAF from spleen Merz et al. (1985) suggested that biochemical differences may
Fig. 2. (a to c) Recovery of SAF proteins from the 18P precipitates and the effect of pK treatment on differentiation of SAF proteins from normal components. Both 18P and 18S were examined for SAF proteins either directly or after treatment with pK (0.1 μg/100 mg tissue). The samples and reagents used were (a) brain samples (140 mg) and peroxidase, (b) spleen samples (300 mg) and (c) lymph node samples (220 mg) and [125I]streptavidin. Lanes 1 and 3 represent 18P treated with pK (ETP); lanes 2 and 4 represent 18P; lanes 5 and 7 represent 18S treated with pK; lanes 6 and 8 represent 18S. Lanes 3, 4, 7 and 8 contain samples derived from roughly equal amounts of the normal mouse tissues. M represents the SAF fraction prepared according to Takahashi et al. (1986). SDS-PAGE was carried out using 15% acrylamide (Laemmli, 1970). Arrowheads indicate Mr (from the top, 50K, 31K, 27.5K, 24.5K, 21K and 17K) estimated from marker proteins of known Mr (cytochrome c monomer and oligomers; Oriental Yeast, Tokyo, Japan). (d and e) Appearance of SAF proteins in brain and spleen following an i.p. inoculation. SAF proteins in the ETP samples prepared from 140 mg of pooled brains (d) were detected with [125I]streptavidin. The same was done with 350 mg (3 to 5 week groups) and 400 to 420 mg (6 to 30 week groups) of pooled spleens (e). Numbers on the top indicate the number of weeks p.i. before mice were sacrificed. Lanes C contain analogous samples from equivalent amounts of the normal mouse tissues. Bars indicate Mr (from the top, 50K, 24.5K, 21K and 17K).
exist between spleen SAF and brain SAF. A large amount of pK-sensitive normal components which share antigenic determinant(s) with SAF proteins were present in 18P and 18S from lymph nodes of both infected and uninfected mice (Fig. 2c, lanes 2, 4, 6 and 8). However, as seen in pK-treated samples from uninfected lymph nodes, they were completely removed by pK treatment (Fig. 2c, lanes 3 and 7). These results indicate that pK treatment is essential to differentiate SAF proteins from normal components that react with the SAF-specific antibodies. We failed to detect SAF proteins in samples prepared from white blood cells even with ultracentrifugation at 305000 g (data not shown).

Next we investigated the time-scale of SAF appearance in brain, spleen and lymph node after i.p. infection. A group of nine mice were sacrificed every week up to 6 weeks and then at 12, 18, 24 and 30 weeks post-infection (p.i.). The onset of clinical symptoms ranged from 21 to 30 weeks p.i.

As shown in Fig. 2(d), SAF proteins were detected in brain samples from the groups sacrificed at 12 (faintly), 24 and 30 weeks p.i. and the concentration of the proteins in the latter two samples was markedly high. The proteins were detected clearly in spleen samples from the groups sacrificed 5 to 30 weeks p.i. and very faintly in the group sacrificed 4 weeks p.i. (Fig. 2e). It has been reported that SAF appear early in low numbers in spleen 20 days following i.p. inoculation and 50 days following i.c. inoculation and that the number of SAF rises during the clinical stage of the disease (Merz et al., 1985). In our study, fluctuations of SAF protein concentration were observed between samples from the groups sacrificed at 5 to 30 weeks p.i. but the marked differences in protein concentration found in the brain samples were not observed in the spleen samples before (weeks 5 to 18 p.i.) and after (weeks 24 and 30 p.i.) the onset of the clinical symptoms. SAF proteins appeared later in the brain samples than in the spleen samples. This is presumably a consequence of the relatively late appearance of infectivity in the central nervous system of i.p. infected mice (Eklund et al., 1967; Cole & Kimberlin, 1985; Kimberlin & Walker, 1979). In lymph node samples, SAF proteins were detected faintly in those from the groups sacrificed at 6, 18 and 24 weeks p.i. (data not shown).

SAF proteins are specifically detected in pK-treated preparations from scrapie-infected, but not uninfected animals (Bendheim et al., 1984; Diringer et al., 1984; Rubenstein et al., 1986; Takahashi et al., 1986). Detection of SAF proteins could therefore be used in the diagnosis of scrapie. We have showed that SAF proteins were detectable by radioimmune WBA using \[^{125}\text{I}\]streptavidin in samples from mouse organs other than brain during the early period of preclinical scrapie infection. If this is the case in sheep, scrapie infection could be diagnosed by detecting SAF proteins during the preclinical stage.

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REFERENCES


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