Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method


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SAF-protein, an amyloid, is the main constituent of scrapie-associated fibrils (SAF) and a specific marker for transmissible spongiform encephalopathies (TSE). Using an improved extraction method and Western blot detection, the disease-specific amyloid was found in various parts of the central nervous system of hamsters orally infected with scrapie, of squirrel monkeys orally infected with kuru, sporadic Creutzfeldt-Jakob disease (CJD) and scrapie, of human patients with sporadic CJD, of a sheep with natural scrapie and of a cow with bovine spongiform encephalopathy (BSE). In human CJD samples, the concentration of TSE-specific amyloid was estimated to be 1000- to 10000-fold lower than in the central nervous system of hamsters with scrapie. The extraction method has a yield of 70% and allows Western blot detection of the TSE-specific amyloid in samples representing 1–10 μg of brain tissue from intracerebrally infected hamsters, as well as in individual spleens from hamsters with terminal scrapie infected by the intracerebral, oral or intraperitoneal route. A 20–100 mg sample of material is sufficient for the extraction of the pathological protein from different rodent, monkey, ovine, bovine and human tissues. The results reported here demonstrate the potential suitability of the method for the routine diagnosis of TSE as well as for the detailed analysis of distribution patterns of the TSE-specific amyloid in experimental approaches to the investigation of these diseases.

Introduction

Scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans are the best known examples of a group of fatal infectious neurodegenerative diseases, the transmissible spongiform encephalopathies, whose causative agent has not yet been identified (for reviews see: Gajdusek, 1990; Carp et al., 1989; Kimberlin, 1990; Rohwer, 1991; Diringer et al., 1994). Scrapie-associated fibrils (SAF) (Merz et al., 1981, 1983, 1984) and prion rods (Prusiner et al., 1982a, 1983) are characteristic morphological features found in TSE-affected tissues under different preparation conditions. They are regarded as different forms of the same structure and do not differ in protein composition or antigenicity (Merz et al., 1987). Both were shown to represent amyloid structures by staining with Congo red (Prusiner et al., 1983) and by infra-red spectroscopy (Caughey et al., 1991; Gasset et al., 1993). SAF consist primarily of the disease-specific SAF-protein [also referred to as protease-resistant protein or prion protein, PrP (Bolton et al., 1982; McKinley et al., 1983)] (Diringer et al., 1983a; Barry et al., 1985; DeArmond et al., 1985; Merz et al., 1987; Wiley et al., 1987). This amyloid protein is derived from a highly conserved host-encoded precursor, a cellular glycoprotein (mol. mass 33–35 kDa, also designated PrPc) (Oesch et al., 1985; Robakis et al., 1986; Locht et al., 1986; Basler et al., 1986; Bolton et al., 1987; Bendheim et al., 1988) located mainly at the surface of nerve cells (Stahl et al., 1987, 1990a, b). During the course of a TSE infection, an as yet unknown post-translational event (Borchelt et al., 1990) converts this cellular protein into a disease-specific pathological isoform (also termed PrPSc) with the same molecular mass (Barry et al., 1986; Meyer et al., 1986; Bolton et al., 1987; Bendheim et al., 1988). Despite their identical amino acid sequence (Stahl & Prusiner, 1991; Stahl et al., 1993), the normal and

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pathological isoforms of the protein differ in their sensitivity towards proteolytic activity.

While the cellular protein is completely digested by proteinase K, the pathological isofrom is transformed into a protease-resistant core of lower molecular mass under non-denaturing conditions. The native pathological protein (mol. mass 33–35 kDa) and its protease-resistant core (mol. mass 27–30 kDa, also referred to as PrP 27-30) are highly aggregated and constitute the amyloid, which presents as SAF in the electron microscope (Bolton et al., 1982, 1987; Diringer et al., 1983a; Diringer, 1992; Meyer et al., 1986). In some cases, this pathological protein, hereafter referred to as disease- or TSE-specific amyloid, forms plaques and can be visualized histologically (Bendheim et al., 1984; DeArmond et al., 1985; Kitamoto et al., 1986). Detection of the TSE-specific amyloid by immunostaining is the most specific criterion for the confirmation of TSE and could, with considerably improved sensitivity, supplement or even replace more traditional diagnostic methods like transmission experiments, infectivity assays (Kimberlin & Walker, 1977; Prusiner et al., 1980, 1982b), electron microscopy for SAF (Merz et al., 1981, 1984; Hope et al., 1988) or histopathology (OIE 1992a, b).

Based on an earlier method using 1% sarcosyl (‘1%-method’) (Diringer et al., 1983b), we have developed a simple and rapid procedure with an improved yield for the extraction of the TSE-specific amyloid from 20–100 mg of tissue from affected individuals of several different species. The ‘1 %-method’ offers an alternative to the ‘10%-method’ published by Oberdieck et al. (1994) for the purification of TSE-specific amyloid, depending on the application. The purified disease-specific amyloid from both protocols can be detected in a Western blot by immunostaining with various available antibodies (for a review see Oberdieck et al., 1994).

In this paper, we describe the application of the improved 1%-method for the detection of disease-specific amyloid in very small amounts of tissue from the central nervous system (CNS) of various species. Further, we were able to detect disease-specific amyloid in individual spleens from orally, intraperitoneally and intracerebrally infected hamsters.

Subsequently, we compared the terminal distribution patterns of disease-specific amyloid in the CNS of hamsters and non-human primates orally infected with TSE, of non-experimentally, but most likely also orally, infected sheep and cattle, and of patients with CJD.

Methods

Tissue samples

(a) Hamster samples. Tissue samples were taken from Syrian hamsters infected orally, intraperitoneally or intracerebrally with scrapie strain 263K at the Robert Koch-Institute, Berlin, Germany. The brain (approximately 1 g of tissue), the vertebral column and the spleen (50–100 mg of tissue) were removed from sacrificed animals. The spinal cord was dissected into segments corresponding to vertebrae C1–C3, C4–C7, T1–T3, T4–T6, T7, T9, T10–T13 and L1–L3 (20–70 mg of tissue each). Equivalent control tissues came from uninfected hamsters.

(b) Squirrel monkey samples. Tissues from the CNS of squirrel monkeys orally infected with Kuru, sporadic CJD and scrapie were analysed at the National Institutes of Health, Bethesda, Md., USA. The frozen brain stems and segments of spinal cord originated from animals infected more than 20 years ago (Gibbs et al., 1980). The original topographic position of the spinal cord segments could not be reconstructed with certainty; however, the range of samples can be assumed to cover the cervical and thoracic regions. For every squirrel monkey, a sample from the brain stem and four different samples from the spinal cord, each containing 70–90 mg of tissue, were analysed.

(c) Human samples. Samples from human patients with sporadic CJD were also analysed at the National Institutes of Health. We investigated 90 mg samples of grey matter from different regions of the spinal cord from four different patients. Again, the original position of the segments within the spinal cord was uncertain. For patient #3, the segments most likely came from the cervical, thoracic and lumbar region.

(d) Ruminant samples. At the Central Veterinary Laboratory in Weybridge, Surrey, UK, tissues taken from the CNS of two ruminants naturally infected with TSE, a Swaledale sheep with scrapie and a cow with BSE, were analysed. Defined segments of the spinal cord were available from both animals. The spinal cord of the sheep was sampled at intervals of about 10–15 cm by dividing it into six segments between the cranial end and the beginning of the cauda equina. A cranial and caudal sample were taken from the first cervical segment, and only a caudal sample from each of the remaining segments. Disease-specific amyloid was extracted from 100 mg of grey matter from each of the spinal cord samples and from 100 mg of thalamus. The extraction of TSE-specific amyloid from a BSE-affected cow was performed with 100 mg of grey matter from each of the spinal cord segments C1, C6, T1, T5, T8, T11, L2 and L5 as well as from 100 mg of tissue from the medulla oblongata.

Extraction of the TSE-specific amyloid. Tissue samples were homogenized with an ultrasonicator in Tris-buffered saline (TBS: 10 mm-Tris–HCl, 133 mm-NaCl, pH 7.4). The homogenates contained tissue concentrations of 20–100 mg per 1 ml. TSE-specific amyloid was extracted from the homogenate following a modification of a previously published protocol (Diringer et al., 1983b). Unless otherwise stated, all centrifugations were carried out at 4 °C, and all remaining steps at room temperature. The rotor used throughout the procedure was a Beckman model TLA-45.

For each sample, 1 ml of homogenate (H) was centrifuged for 3 min at 5000 r.p.m. The supernatant was removed to a polyallomer rotor tube (Beckman), the pellet resuspended in 0.5 ml of TBS and centrifuged again as before. The resulting supernatant was added to the first supernatant, and the pellet (P1) discarded.

The combined supernatant (S1) was centrifuged for 40 min at 20000 r.p.m. The supernatant (S2) was then discarded, and the pellet (P2) suspended in 1 ml 1% sarcosyl in TBS. The suspension was centrifuged for 2.5 h at 45000 r.p.m. The next supernatant (S3) was discarded and the pellet (P3) resuspended in 1 ml 0.5% sarcosyl, 10% (w/v) NaCl in TBS, with proteinase K added to a final concentration of 2.5 μg/ml.

After a final centrifugation at 45000 r.p.m. for 2.5 h, the supernatant (S4) was discarded. The pellet (P4) contained the TSE-specific amyloid.

In order to evaluate the purification protocol, three brains from intracerebrally infected hamsters with terminal scrapie were each
homogenized in 10 ml of TBS. From each brain homogenate, 1 ml containing 100 mg of brain tissue was subjected to the extraction procedure. Aliquots from all important fractions were removed for further analysis.

**Preparation of homogenates from scrapie-infected and normal hamster brains for immunoblotting without prior purification.** For determination of TSE-specific amyloid directly from homogenate of hamster brains, 50 μl of homogenate (corresponding to 5 mg of brain tissue) was incubated for 1 h at 37 °C with 6 μl 11% (w/v) sarcosyl in TBS and 10 μl of a 1 mg/ml solution of proteinase K. The digestion was stopped by adding 65 μl of doubly concentrated sample buffer [i.e. 4% (w/v) SDS 10% (v/v) 2-mercaptoethanol in 120 mm-Tris–HCl, pH 6.8, containing 20% (w/v) glycerol and 0.05% (w/v) bromophenol blue] and boiling for 5 min.

Normal precursor protein in the brain and spinal cord from control hamsters was visualized by diluting 50 μl of unprocessed homogenate (containing 1–5 mg of tissue) in 50 μl of doubly concentrated sample buffer followed by blotting defined aliquots of the mixture.

**SDS-disc PAGE, silver staining and immunoblot.** The purified samples from hamsters, monkeys and patients with sporadic CJD as well as the homogenates prepared for immunoblotting without prior purification were run on 12.5% minigels (Mini-protein II Dual Slab Cell, Bio-Rad) following an SDS-disc PAGE protocol (Laemmli, 1970). The gels were then either silver-stained following the Oakley procedure (Oakley et al., 1980) or blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) with the semi dry method (Burnette, 1981; Tovey & Baldo, 1987) using the Fast-Blot system (Biometra). Blots were stained following a modification of a previously published procedure (Oberdieck et al., 1994). The primary antibody MAb 3F4 (Kascak et al., 1987) directed against the epitope Met-Lys-His-Met of the amyloid protein (Lowenstein et al., 1990; Rogers et al., 1991; Bolton et al., 1991b) was used in combination with a biotin–streptavidin-based enhancement kit (Dako). TSE-specific amyloids from hamster, non-human primates and humans share the epitope recognized by MAb 3F4 (Basler et al., 1986; Liao et al., 1986). This allowed us to use the same immunological detection protocol for the analysis of the CNS of squirrel monkeys, human samples and hamster tissues.

As neither the ovine nor the bovine TSE-specific amyloid harbours the complete epitope for MAb 3F4 (Goldmann et al., 1990, 1991), we resorted to the polyclonal antibodies SP40 (generous gift of B. Anderson, Institute of Psychiatry, London, UK) for sheep and IB3 (generous gift of C. Farquhar, AFRC/MRC Neuropathogenesis Unit, Edinburgh, UK) for cows (Langeveld et al., 1994). SDS-disc PAGE and development of the immunoblots were carried out with the following modifications. Samples were loaded onto large format gels (approximately 18 × 15 cm) of the BRL vertical electrophoresis system and wet blotted (Towbin et al., 1979) onto PVDF membranes using the Bio-Rad Transblot cell. Immunostaining was carried out with polyclonal antisera IB3 and SP40 raised in rabbits at a dilution of 1:1000. The TSE-specific amyloid was detected with an anti-rabbit gold probe according to the Auroprobe BL plus kit enhanced with the Intense kit (both from Cambio).

**Preparation of a highly purified standard of TSE-specific amyloid.** A defined standard containing a known amount of highly purified TSE-specific amyloid was prepared from brains of hamsters with terminal scrapie following a modified version of a method published earlier (Diringer et al., 1983b), in that no nuclease or protease digestion was performed up to P25, was prepared directly from Sk and dissolved in 50 ml 1% (w/v) sarcosyl–TBS. P31 was suspended in a total of 25 ml 1% (w/v) sarcosyl–10% (w/v) NaCl–TBS. P31 was pooled in a total of 1 ml 0.1% (w/v) sarcosyl–TBS and stirred overnight at 4 °C. The suspension was transferred to a polyallomer centrifuge tube (Beckman), and proteinase K was added to a final concentration of 1 μg/ml. After incubating for 60 min at 37 °C, the solution was centrifuged in a Beckman TLA-45 rotor for 20 min at 13000 r.p.m. The pellet was suspended in sample buffer, boiled for 5 min and subjected to preparative SDS-disc PAGE on 12.5% gels in an electrophoresis apparatus from GIBCO/BRL (model 1100 PG) according to the manufacturer’s guidelines. Eluted fractions containing TSE-specific amyloid (mol. mass 20–30 kDa) were pooled. SDS-disc PAGE and subsequent silver staining (Oakley et al., 1980) revealed no contamination (data not shown). The amount of total protein in the purified standard equals the amount of TSE-specific amyloid and was determined as described below.

**Determination of total protein content.** The total protein content in a sample was determined by staining with amido black (Neuhoff et al., 1979; Nakamura et al., 1985). The samples were dissolved in sample buffer, boiled for 5 min and applied to a PVDF membrane in a Bio-Dot microfiltration apparatus (Bio-Rad). A standard curve was produced from samples containing defined amounts (20–200 ng) of bovine serum albumin. The intensities of the stained spots were determined with a densitometry programme (CSI Videodevice and CAM software; Cybertech, Berlin, Germany). The staining intensities of unknown samples were correlated to their protein contents using the standard curve.

**Quantification of the TSE-specific amyloid.** For quantification of the amount of TSE-specific amyloid, the blot signals of unknown samples were recorded with a densitometry programme (CSI Videodevice and CAM software; Cybertech) and their intensities compared to those of reference signals on the same blot. Reference signals were generated from the highly purified standard with known amounts of TSE-specific amyloid.

The immunological detection system based on MAb 3F4 and signal enhancement with biotin–streptavidin allows detection of as little as 0.2–0.1 ng of our highly purified standard of TSE-specific amyloid in the Western blot (data not shown). It is assumed that samples producing blot signals of equal intensities contain equal amounts of antigen, i.e. TSE-specific amyloid, provided they were stained on the same membrane under identical conditions. This holds true if the blotted antigens do not differ in their affinity towards the primary antibody used for detection and should not present a problem for TSE-specific amyloid from different individuals of the same species.

Under the conditions described above, a sample giving rise to a blot signal of the same intensity as a certain amount of the standard is assumed to contain the same amount of TSE-specific amyloid as this reference sample. The correlation between the amount of amyloid and signal intensity follows a regression curve of the second order and does not reach saturation over the range 0.1–20 ng of TSE-specific amyloid.

**Results**

**Evaluation of the method**

For an evaluation of the purification protocol, the amount of total protein and of disease-specific amyloid was determined in all important fractions (Table 1). Some of these fractions were also analysed by SDS-disc PAGE followed by silver staining (Fig. 1a) and immunoblotting (Fig. 1b). The TSE-specific amyloid does not stain well with silver (Fig. 1a, lane 5), and even concentrated samples appear only as a diffuse shadow (Fig. 1a, lane 6). In the immunoblot, by contrast, a characteristic three-band pattern in the molecular mass range 20–30 kDa can be clearly visualized.
Table 1. Extraction of TSE-specific amyloid from hamster brains

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein* (mg)</th>
<th>TSE-specific amyloid* (μg)</th>
<th>Specific concentration†</th>
<th>Purification factor</th>
<th>Recovery (%)</th>
</tr>
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<tr>
<td>Homogenate</td>
<td>100 ± 15</td>
<td>100 ± 10</td>
<td>0.0010</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S1</td>
<td>80 ± 10</td>
<td>90 ± 15</td>
<td>0.0011</td>
<td>1-1</td>
<td>90</td>
</tr>
<tr>
<td>P2</td>
<td>35 ± 5</td>
<td>80 ± 15</td>
<td>0.0023</td>
<td>2-3</td>
<td>80</td>
</tr>
<tr>
<td>P3</td>
<td>43 ± 1.5</td>
<td>75 ± 10</td>
<td>0.0174</td>
<td>17-4</td>
<td>75</td>
</tr>
<tr>
<td>P4</td>
<td>0.28 ± 0.1</td>
<td>70 ± 10</td>
<td>0.2500</td>
<td>250</td>
<td>70</td>
</tr>
</tbody>
</table>

* Amounts per gram of tissue. Means calculated from three preparations starting with 100 μg each of TSE-specific amyloid in the homogenate.
† Ratio between the amount of TSE-specific amyloid and the amount of total protein.

The amount of TSE-specific amyloid in brain homogenates from terminally ill hamsters varied between 70 and 110 μg per gram of brain tissue. With 100 μg of disease-specific amyloid in the starting homogenate, 60–80 μg of amyloid were retrieved in the final pellet, which represents a mean yield of about 70%. The yield remains constant for samples of between 20 and 100 mg of tissue (data not shown). A comparison of the amount of total protein and TSE-specific amyloid in the final pellet revealed a purity of 25%. As MAb 3F4 shows insignificant cross-reactivity with other proteins, further purification would not justify the concomitant reduction in yield. Control samples corresponding to up to 0.1 g of tissue gave no signal in a Western blot (data not shown).

Individual spleens (50–100 mg of tissue) of terminally ill hamsters infected with scrapie by the oral, intraperitoneal or intracerebral route were subjected to the extraction procedure. Three individual spleens were analysed for each route of infection. As shown in Fig. 2, disease-specific amyloid could be detected in the Western blot from nine-tenths and, with the exception of the intracerebral infection, from one-tenth of the final pellet (representing 5–10 mg of tissue).

### TSE-specific amyloid from hamster spleens

Individual spleens (50–100 mg of tissue) of terminally ill hamsters infected with scrapie by the oral, intraperitoneal or intracerebral route were subjected to the extraction procedure. Three individual spleens were analysed for each route of infection. As shown in Fig. 2, disease-specific amyloid could be detected in the Western blot from nine-tenths and, with the exception of the intracerebral infection, from one-tenth of the final pellet (representing 5–10 mg of tissue).
The TSE-specific amyloid and its normal precursor protein in the CNS of hamsters

The specific blot signals resulting from 20–50 µg of brain and segmented spinal cord from an orally infected hamster are shown in Fig. 3(a). The brain and six different spinal cord segments from between vertebrae C1 and T13 gave rise to signals of comparable intensity. Thus, the concentration of disease-specific amyloid appeared to lie within a similar range in these samples. In contrast, the amount of TSE-specific amyloid in the lumbar sample (L1–L3) was significantly lower. Here, the blot signal resulted from 20 mg of tissue, compared to 20–50 µg of tissue for the other spinal cord samples.

The distribution pattern of the cellular precursor protein in the CNS of healthy hamsters is shown in Fig. 3(b). The intensities of the blot signals from the brain sample and from spinal cord samples between C1 and T13, each representing 100 µg of tissue, do not differ significantly. Again, the signal from lumbar segment L1–L3 is less intense. Thus, the lumbar region differs from the remainder of the spinal cord in that it contains lower levels both of the normal precursor protein and the disease-specific amyloid.

Disease-specific amyloid in the spinal cord and brain of primates

Each blot signal of the samples from squirrel monkeys (Fig. 4a–c) represents between 70 and 90 mg of tissue. All samples from the animal infected with CJD gave rise to strong immunostaining signals of similar intensities (Fig. 4a). The same applied for the monkey infected with scrapie (Fig. 4b). Only one spinal cord segment, sample 2, deviated with a clearly weaker signal.

A different result was obtained for the squirrel monkey infected with kuru (Fig. 4c), where the Western blot showed a remarkable variation in the amount of disease-specific amyloid between the samples. A strong signal was again seen for the brain stem, while the spinal cord samples, other than those of the monkeys infected with CJD or scrapie, gave only weak signals. The result for sample 3 was negative.

The Western blots of the human tissue samples are shown in Fig. 5. Strong signals clearly indicated the presence of TSE-specific amyloid in all samples. As determined from comparative dilution series with the highly purified standard of TSE-specific amyloid from hamsters (data not shown), the variation in signal intensities corresponds to an approximately tenfold difference in the amount of antigen.

TSE-specific amyloid in the spinal cord and brain of a sheep with scrapie and a cow with BSE

Fig. 6 shows the results of the analysis of the sheep material. The entire pellets from the extraction procedure, each representing 100 mg of tissue, were subjected to Western blot analysis. All spinal cord samples from the cervical region to the cauda equina clearly showed the immunostaining characteristic of the disease-specific amyloid. The sample from the thalamus gave a weak, barely detectable signal.

Similarly, disease-specific amyloid was detected in the brain and spinal cord from the cow with BSE (Fig. 7). The entire final pellets, each representing 100 mg of tissue from spinal cord segments C1, C6, T1, T5, T8, T11, L2 and L5 as well as from the medulla oblongata were applied to the blot. The lowest concentrations of TSE-specific amyloid were found in positions T5 and T8 and the highest concentration in C6.
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Fig. 4. Western blot detection of TSE-specific amyloid in P4 from the CNS of squirrel monkeys orally infected with sporadic CJD (a), scrapie (b) and kuru (c). Immunostaining was performed with MAb 3F4. Brain stem (lane 1) and samples of segmented spinal cord (lanes 2–5). Samples represent between 70 and 90 mg of tissue.

Fig. 5. Western blot detection of TSE-specific amyloid in P4 from the spinal cord of patients who died of sporadic CJD. Sample from patient #1 (lane 1); samples from patient #2 (lanes 2–4); samples from patient #3 (lanes 5–7) probably covering cervical, thoracic and lumbar regions of the spinal cord; sample from patient #4 (lane 8). All spinal cord samples represent 90 mg of grey matter.

Discussion

The spread of the agent causing TSE from peripheral organs to the brain of infected individuals is of central importance for an understanding of the pathogenesis of TSE. Major contributions to this area have been made by R. H. Kimberlin and C. A. Walker (Kimberlin, 1979; Kimberlin & Walker, 1979, 1982, 1983, 1986). However, for the oral route of infection, the most relevant pathway to natural disease in animal TSE, this aspect has previously received little attention in infectivity studies (Kimberlin & Walker, 1989b) and has not been approached at all with the tools of protein analysis.

The techniques described in this paper were applied in a first attempt to map the terminal distribution pattern of disease-specific amyloid in the CNS, particularly in the spinal cord, of sheep and cows most likely infected orally, of monkeys orally infected with human TSE and scrapie, and of patients with sporadic CJD.

The starting point for our study was the analysis of the distribution pattern of the disease-specific amyloid in the CNS of orally infected hamsters. In these rodents, the lumbar segment L1–L3 showed approximately 500- to 1000-fold less amyloid than the remaining samples. Whether this is due to the lower amount of the cellular precursor protein in the lumbar segment and possible implications for the mechanism of pathogenesis will be addressed in future experiments.

Using the same immunological detection system, 70–90 mg of tissue from squirrel monkeys orally infected with scrapie or sporadic CJD produced blot signals with
Western blot mapping of amyloid in TSE

Fig. 6. Western blot detection of disease-specific amyloid in P4 from the CNS of a Swaledale sheep affected with natural scrapie. Sample representing 100 mg of thalamus (lane 1); spinal cord samples each representing 100 mg of grey matter (lanes 2–7). The spinal cord was divided into six approximately equal segments between the cranial end and the beginning of the cauda equina. The first, cervical segment was sampled at its cranial (lane 2) and caudal end (lane 3), the remaining segments at their caudal end only (lanes 4–7). Immunostaining was performed with polyclonal antiserum SP40.

Fig. 7. Western blot detection of disease-specific amyloid in P4 from the CNS of a BSE-affected cow. Sample representing 100 mg of medulla oblongata (lane 1). Spinal cord samples each representing 100 mg of grey matter taken from the following vertebrae: C1 (lane 2), C6 (lane 3), T1 (lane 4), T5 (lane 5), T8 (lane 6), T11 (lane 7), L2 (lane 8) and L5 (lane 9). Immunostaining was performed with polyclonal antiserum 1B3.

intensities between those of samples representing 20–50 µg (Fig. 3a) and 200–500 µg (data not shown) of tissue from the brains or spinal cord segments C1–T13 from terminally ill hamsters. Assuming a comparable affinity of the disease-specific amyloid protein from both species for MAB 3F4, this indicates an approximately 200–2000-fold lower concentration of the TSE-specific amyloid in neural tissues from monkeys than in the corresponding samples from hamsters. For the squirrel monkey infected with Kuru, the amount of amyloid in the spinal cord appeared to be even lower, as the signals resulting from equivalent samples were still weaker and included one negative result. The low amount of disease-specific amyloid in the lumbar segment of hamster spinal cord allows speculation as to whether the negative monkey sample might also originate from the lumbar region.

For the four different human cases of CJD, all samples taken from the spinal cords were positive by Western blot for disease-specific amyloid. The signal intensities imply a 1000–10000-fold lower concentration of TSE-specific amyloid than in comparable hamster samples. It should be kept in mind that only the grey matter from the human tissue samples was used, whereas the entire tissue segment was extracted for the hamsters and monkeys.

As the detection of TSE-specific amyloid in ovine and bovine tissues required polyclonal antibodies and a different staining procedure, we did not attempt a quantitative comparison of the resulting signal intensities in the Western blot with those obtained in the previous experiments. However, the TSE-specific amyloid was present in all of the samples (representing 100 mg each of grey matter) from the CNS of both the scrapie-affected sheep and the BSE-affected cow.

It remains to be investigated whether the varying intensities, particularly for the signals from the bovine samples, result from tissue sampling, or whether they can be confirmed with homogenates of larger portions of tissue.

We have determined the amount of disease-specific amyloid in proteinase K-treated homogenates of brains from hamsters with terminal scrapie as 70–110 lag per brain. Our results differ widely from those of Prusiner et al. (1990), who report only 7.2 lag of TSE-specific amyloid per brain. However, they are comparable to those of Bolton et al. (1987, 1991a), who report 25–80 µg of disease-specific amyloid per gram of tissue in purified fractions from hamster brains containing 15–30% of the scrapie infectivity in the starting material.

On this basis Bendheim et al. (1988) estimate the total amount of TSE-specific amyloid in a scrapie-affected hamster brain to be about 60–160 µg. In a recent publication Rubenstein et al. (1994) report the puri-
fication of TSE-specific amyloid with a yield of 30–40 μg per gram of brain tissue.

The amount of TSE-specific amyloid in the brains of hamsters with terminal scrapie corresponds to an infectivity of about 10^8–10^9 LD_50 (Czub et al., 1986b, 1988; Bolton et al., 1991; Rubenstein et al., 1991). The sensitivity of our method allows a quantitative comparison of the time-dependent development of both infectivity and disease-specific amyloid in hamsters over a range of about 10^−3–10^0 LD_50 and 0·01–100 μg of TSE-specific amyloid per gram of tissue. This constitutes a considerable improvement in accuracy as compared to earlier pathogenesis studies performed by Czub et al. (1986b, 1988) (unpublished work).

The detection of TSE-specific amyloid from hamster spleens in the Western blot has previously been accomplished only after pooling samples from at least 25 animals, due to the low concentration of amyloid in the spleen (Rubenstein et al., 1991; Kimberlin & Walker, 1989a). When less sensitive procedures were employed, they produced negative results (Czub et al., 1986a). The improved method allows the study of individual spleens starting early in the disease process. Rubenstein et al. (1991) estimate the concentration of TSE-specific amyloid in the spleen of an intracerebrally infected hamster with terminal scrapie to be 5000- to 10000-fold less than in the brain. The detection limits we found for TSE-specific amyloid from brain (equivalent to 1–10 μg of tissue) and spleen (equivalent to 5–10 mg of tissue) reflect a difference of a comparable order of magnitude.

The results presented here demonstrate the suitability of our method for the routine diagnosis of TSE in non-experimental hosts such as sheep, cows or humans. The protocol allows the reliable detection of the disease-specific amyloid in 100 mg or less of grey matter from different positions in the spinal cord of these species and can be applied to the screening of large numbers of samples. It provides a specific diagnosis of TSE with modest requirements for equipment and experimental experience. Improved antibodies against proteinase K-treated ovine and bovine TSE-specific amyloid could further enhance the sensitivity of the test (Farquhar et al., 1989, 1994).

In the future, precise mapping of the terminal distribution pattern of TSE-specific amyloid in the CNS and in peripheral organs of different species following various routes of infection could open new perspectives in the diagnosis and study of TSE. This approach may lead to the localization of the points of entry of the infectious agent into the CNS in experimental as well as in natural disease (Kimberlin & Walker, 1979, 1983, 1986). Ideally, distinct terminal distribution patterns might even allow a post-mortem identification of the route of infection.

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