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Technical Advance

The Paraffin-Embedded Tissue Blot Detects PrP^{Sc} Early in the Incubation Time in Prion Diseases

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With the appearance of bovine spongiform encephalopathy (BSE) and a new variant of Creutzfeldt-Jakob disease (nvCJD) that seems to be caused by BSE, there is an increased need for improvement of diagnostic techniques and recognition of all variants of prion diseases in humans and animals. Publications on the immunohistochemical identification of PrP^{Sc} in the tonsils and appendix in the incubation period of nvCJD indicate that new and more sensitive techniques for the detection of PrP^{Sc} in various tissues may be a valuable tool for early diagnosis in prion diseases. We developed a new and sensitive technique to detect PrP^{Sc} in formalin-fixed and paraffin-embedded tissue, the paraffin-embedded tissue blot (PET blot), and reinvestigated archival brain material from CJD as well as BSE and scrapie. In addition, C57/Bl6 mice experimentally infected with the ME7 strain were investigated sequentially during the incubation time to compare this new technique with conventional methodologies. The PET blot detects PrP^{Sc} in idiopathic (sporadic) and acquired prion diseases, even in cases with equivocal or negative immunohistochemistry, and is more sensitive than the conventional Western blot and histoblot techniques. The PET blot makes possible the detection of PrP^{Sc} during the incubation period long before the onset of clinical disease and in prion disease variants with very low levels of PrP^{Sc}. In mice experimentally infected with the ME7 strain, the PET blot detects PrP^{Sc} in the brain 30 days after intracerebral inoculation–145 days before the onset of clinical signs. Its anatomical resolution is superior to that of the histoblot technique. It may therefore be of particular interest in biopsy diagnosis. Thus it complements other tissue-based techniques for the diagnosis of prion diseases in humans and animals. ***(Am J Pathol 2000, 156:51–56)***

Prion diseases such as Creutzfeldt-Jakob disease (CJD) in humans and scrapie in sheep have been known as rare neurodegenerative and transmissible diseases for many years. With the appearance of bovine spongiform encephalopathy (BSE) and a new variant of Creutzfeldt-Jakob disease (nvCJD) that seems to be caused by BSE,[1] there is an increased need to improve diagnostic methods and to recognize all variants of prion diseases in humans and animals.

The definite diagnosis of prion diseases is based on histological investigations of brain tissue or the biochemical detection of the protease-resistant isoform of the prion protein (PrP^{Sc}).[2] In idiopathic (sporadic) CJD PrP^{Sc} has been detected exclusively in tissues of the central nervous system. In sheep with scrapie and patients of nvCJD, however, PrP^{Sc} has been detected in the tonsils.[3,4] In one case of nvCJD PrP^{Sc} was identified in the appendix 8 months before the onset of clinical disease.[5]

Three techniques with various inherent advantages and disadvantages are used for PrP^{Sc}-based diagnosis of CJD, BSE, and scrapie: 1) Immunohistochemistry, which is highly sensitive, shows superior anatomical resolution and can be performed on formalin-fixed and paraffin-embedded material. 2) The Western blot technique may be more sensitive and has the additional advantage of showing the different PrP^{Sc} types in CJD.[6] Western blots require unfixed tissue and reveal no anatomical details. 3) The histoblot technique combines sensitive protein detection with anatomical tissue preservation. It has been used extensively in prion research; it seems useful for the detection of small amounts of PrP^{Sc} in brain tissue and is believed to be even more sensitive than Western blots.[7,8] A definite disadvantage of the histoblot technique is its requirement for unfixed material; in the

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vast majority of routine and archival cases only formalin-fixed tissue is available in human and veterinary pathology.

We reasoned that by combining particular technical aspects of immunohistochemistry with the histoblot technique it might be possible to increase the sensitivity while preserving satisfactory anatomical detail. Here we present the results of this new technique, the paraffin-embedded tissue blot (PET blot), which has advantages over conventional immunohistochemical, Western blot, and histoblot techniques.

Materials and Methods

Materials

Formalin-fixed and unfixed frozen brain tissue was taken from a large CJD surveillance study in Germany. In CJD cases the PrP gene (*PRNP*), including the polymorphism for methionine (M) and valine (V) at codon 129, was characterized.[9] PrP^{Sc} types were investigated in 31 cases as described by Parchi et al.[6] The PET blot technique was performed on 42 cases of sporadic CJD (sCJD) and included all variations of the polymorphism at codon 129 of *PRNP*, MM homozygotes (15 cases), MV heterozygotes (13 cases), and VV homozygotes (14 cases). All combinations of these three genotypes with PrP^{Sc} types 1 and 2 were investigated. The 24 controls were Alzheimer disease (12 cases), multiinfarct dementia (three cases), cerebral lymphoma (three cases), hypoxic encephalopathy (two cases), and one case each of bacterial meningitis, dementia with Lewy bodies, frontotemporal dementia, and Alper disease. For comparative studies the PET blot was used to investigate various prion diseases of animals. Weanling mice were intracerebrally infected with the ME7 scrapie strain and sacrificed sequentially at days 30, 60, 90, 120, and 150 and at the terminal stage 177 days after infection. In addition, a small number of brains from BSE-infected cattle and scrapie-infected sheep were investigated. Normal animal brains were used as controls.

PET Blot

Formalin-fixed brain tissue was cut into 2-mm-thick tissue blocks, decontaminated in concentrated formic acid for 1 hour, postfixed in 4% phosphate-buffered saline-buffered formalin for 48 hours following the protocol of Brown et al,[10] and embedded in paraffin. Sections (5–7 μm) were cut on a microtome, placed in a water bath (55°C), collected on a prewetted 0.45-μm-pore nitrocellulose membrane (Bio-Rad, Richmond, CA), and dried for at least 30 minutes at 55°C. The nitrocellulose membrane was deparaffinized with xylene. Xylene was replaced with isopropanol, followed by stepwise rehydration. Tween 20 was added at a final concentration of 0.1% to the last rehydration step in distilled H_2O. Membranes were dried and stored at room temperature for months without loss of quality of subsequent PrP^{Sc} staining.

After prewetting with TBST (10 mmol/L Tris-HCl, pH 7.8; 100 mmol/L NaCl; 0.05% Tween 20) digestion was performed with 250 μg/ml proteinase K (Boehringer) in PK-buffer (10 mmol/L Tris-HCl, pH 7.8; 100 mmol/L NaCl; 0.1% Brij 35) for 8 hours at 55°C. With this step the membrane-attached proteins were fixed to the membrane. After washing three times with TBST, the proteins on the membranes were denatured with 3 mol/L guanidine isothiocyanate in 10 mmol/L Tris-HCl (pH 7.8) for 10 minutes. Guanidine was washed out three times with TBST. Immunodetection was performed after preincubation in blocking solution (0.2% casein in TBST) for 30 minutes. The monoclonal antibodies 3F4 (1:2500),[11] Gö138 (1:300),[2] 3B5 (1:50), and 12F10 (1:50)[12] were used as primary antibodies in the blocking solution. Incubation was for at least 1 hour. After three washes in TBST, incubation for at least 1 hour was performed with an alkaline phosphatase-coupled rabbit anti-mouse antibody (Dako, Hamburg) at a dilution of 1:500. After five washes in TBST for 10 minutes, the membranes were adjusted to alkaline pH by incubating two times for 5 minutes in NTM (100 mmol/L Tris-HCl, pH 9.5; 100 mmol/L NaCl; 50 mmol/L $MgCl_2$). The visualization of the antibody reaction was provided by formazan reaction using NBT/BCIP. Blots were evaluated with an Olympus dissecting microscope.

Histoblot

Ten-micron frozen sections were cut on a Reichart-Jung cryomicrotome, mounted on nitrocellulose membranes (0.45-μm pore size; Bio-Rad), and lysed in a buffer containing 10 mmol/L EDTA, 0.5% Na-desoxycholate, and 0.5% Nonidet P-40 in 10 mmol/L Tris-HCl and 100 mmol/L NaCl. Proteinase K digestion was performed at 55°C for 8 hours with 50 μg/ml proteinase K (Boehringer). Proteins were denatured using 3 mol/L guanidine isothiocyanate. Immunodetection was performed as described above.

Immunohistochemistry

Immunohistochemical staining of 2-μm microtome sections was performed after hydrolytic autoclaving in 2 mmol/L HCl at 121°C for 30 minutes.[13] After incubation with the primary antibody (either Gö 138, 3F4 or 12F10) at 4°C for 18 hours a standard APAAP technique was used for detection. The slides were counterstained with hemalum.

Western Blot

Tissues were homogenized in 9 volumes of lysis buffer (100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/L Tris, pH 7.4). Aliquots were digested with proteinase K at a concentration of 50 μg/ml at 37°C for 1 hour. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride (2 mmol/L final concentration). Samples were boiled in elec-

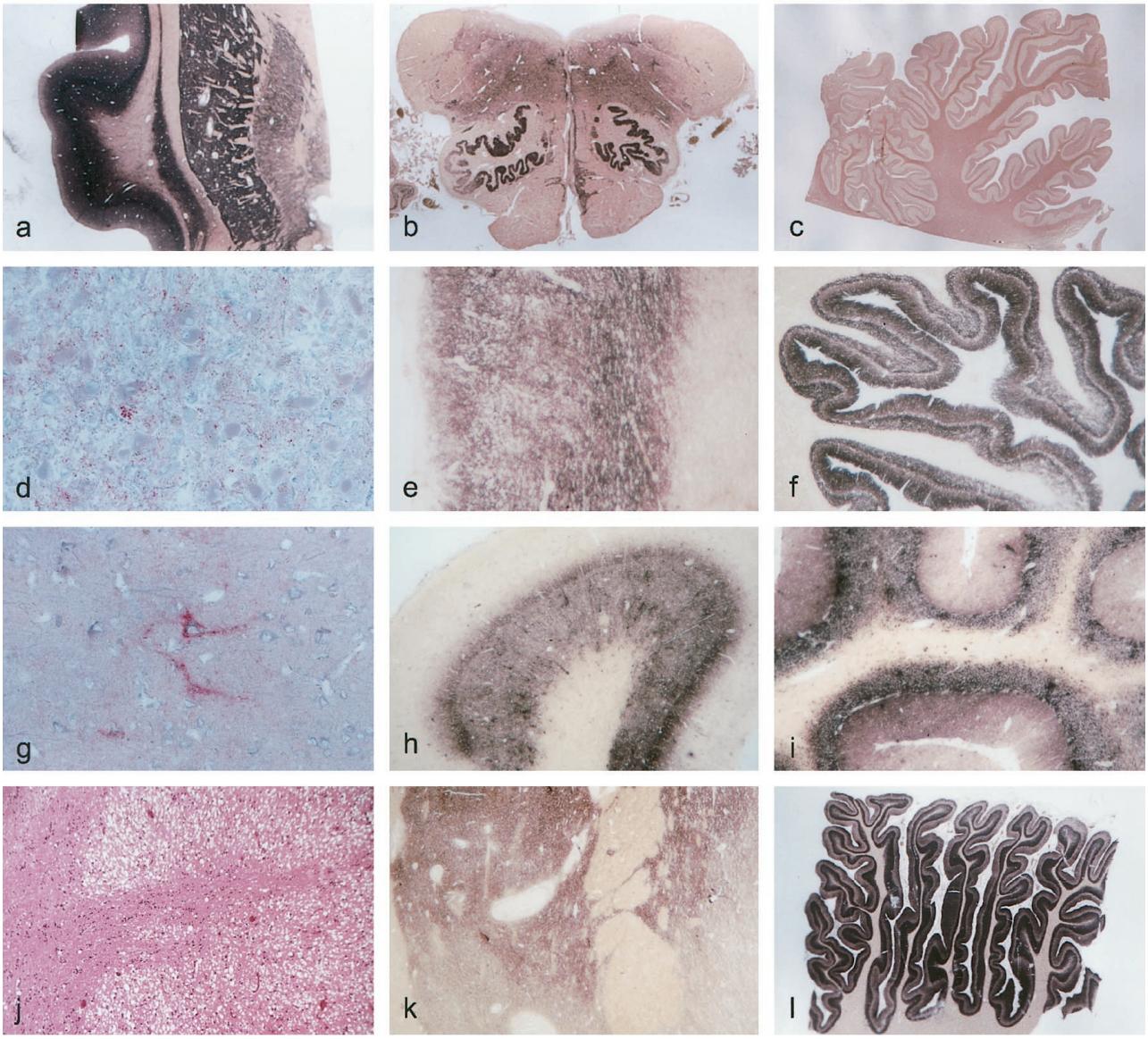
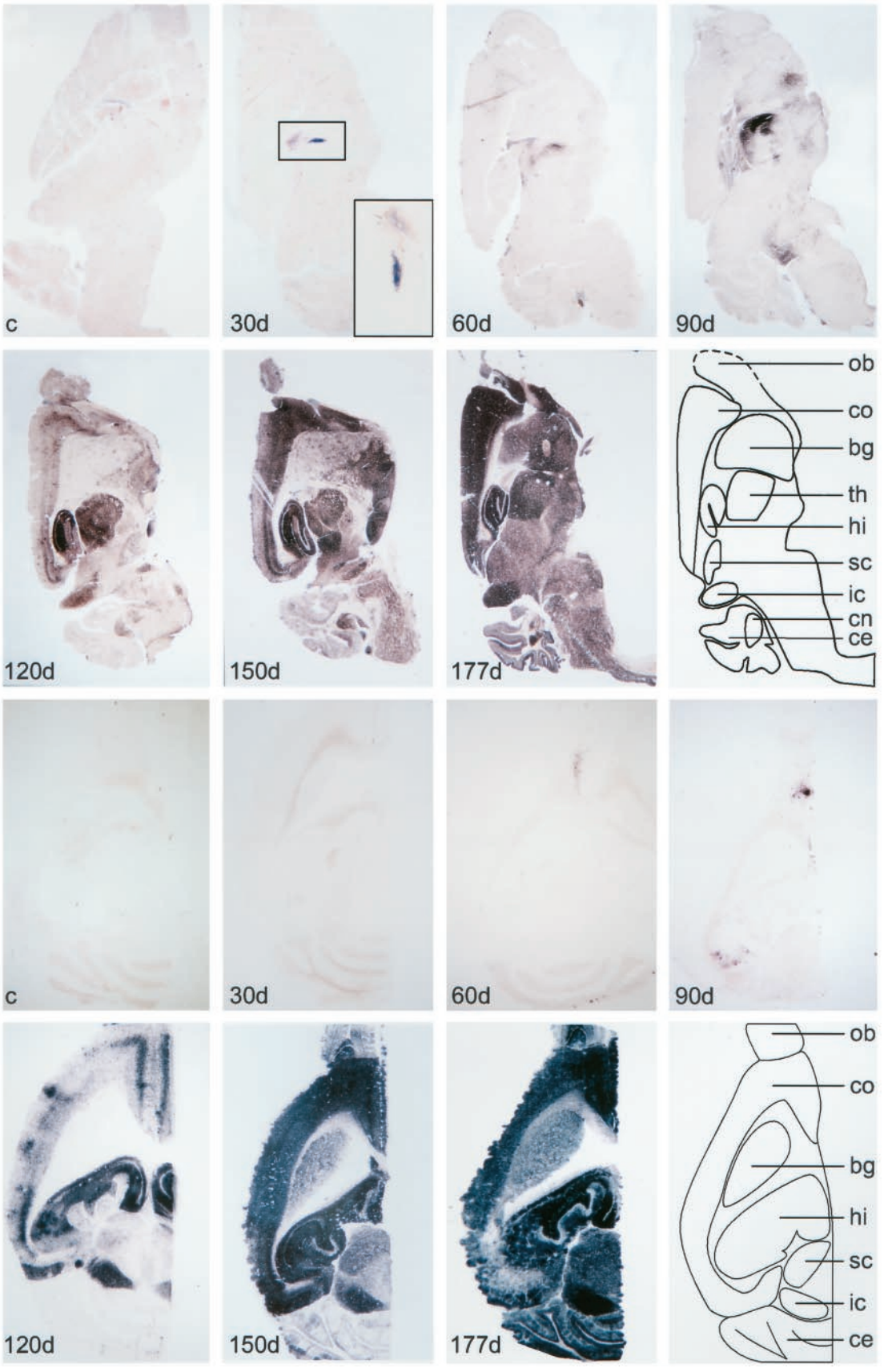


Figure 1. Histology, immunohistochemistry, and PET blot of human sporadic CJD. **a:** PET blot of insular cortex and basal ganglia (VV, PrP^{Sc} type 2). There is very intense staining of the cortex, claustrum, and putamen; less intense staining of the globus pallidus; and no staining of the white matter (primary antibody 12F10). **b:** PET blot of medulla oblongata (MV, PrP^{Sc} type 2). Very strong staining of the inferior olivary nucleus is observed in many cases of sporadic CJD. The raphe nuclei and otherbrain stem nuclei stain less intensely (primary antibody 3F4). **c:** PET blot of the cerbellum of a control case (Alzheimer's disease). Virtually no staining is noted in control cases of patients with other brain diseases (primary antibody 3F4). **d–f:** Sporadic CJD, MM PrP^{Sc} Type 1. **d:** Immunohistochemistry shows very delicate punctate (synaptic) staining. Primary antibody 12F10. Magnification, ×400. **e:** A reticular staining pattern is seen in all cortical layers in a PET blot from the neocortex (same tissue block as in **d**). Primary antibody 12F10. Magnification, ×110. **f:** In the cerebellum there is strong staining in the internal granular layer and somewhat fainter staining in the molecular layer. There is no positivity in the white matter or in the Purkinje cell layer. Primary antibody 12F10. Magnification, ×50. **g–i:** Sporadic CJD, VV PrP^{Sc} Type 2. **g:** Immunohistochemistry shows strong pericellular staining for PrP^{Sc} in the neocortex. Primary antibody 3F4. Magnification, ×400. **h:** A PET blot of the neocortex demonstrates strong pericellular and faint granular PrP^{Sc} deposition in the lower layers. The upper cortical layers, as in many cases of this type, and the subcortical white matter show some granular staining. Primary antibody 3F4. Magnification, ×50. **i:** The cerebellum shows faint staining in the molecular layer and strong granular staining in the granular layer corresponding to plaque-like PrP deposition in immunohistochemistry. As has been noted with immunohistochemistry, small plaque-like deposits are also seen in the white matter of the cerebellum in VV type 2 cases. Primary antibody 3F4. Magnification, ×65. **j–l:** Sporadic CJD, VV PrP^{Sc} Type 1. **j:** Small, densely packed vacuoles are seen on an H&E-stained section of the basal ganglia. In this case PrP^{Sc} could not be visualized using conventional immunohistochemistry. Magnification, ×200. **k:** The PET blot of this case demonstrates strong and unequivocal PrP^{Sc} staining in the basal ganglia. Note that the white matter (internal capsule) in this section is completely negative. Primary antibody 3F4. Magnification, ×25. **l:** PET blot of the cerebellum of the same case shows strong positivity in the internal granular layer and some positivity in the molecular layer. Primary antibody 3F4.

trophoresis buffer (3% sodium dodecyl sulfate in 60 mmol/L Tris, pH 6.8) and run on a 12% sodium dodecyl sulfate-polyacrylamide gel. Each lane contained the equivalent of 1 mg of tissue or 0.25 μg total protein from the lysate. Western blotting was performed by semidry transfer to a nitrocellulose membrane (0.45 μm; Bio-Rad), and immunodetection was performed using the polyclonal antibody K72,[14] followed by goat anti-rabbit IgG (Dako) coupled to alkaline phosphatase. Enzymatic activity was visualized using the CDP-Star chemiluminescent system (Tropix, Bedford, MA) as described by the manufacturer.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Results

In this study of 42 confirmed cases of human sporadic CJD, the PET blot showed excellent anatomical detail and moderate to strong PrP^{Sc} staining (Figure 1). Brain tissue from 24 human controls with various diseases was used to test the specificity of the technique. There was no staining in any of these 24 cases. In sCJD the PET blot shows PrP^{Sc} in the cortex, basal ganglia, brainstem, and areas such as the inferior olive, where this is notoriously difficult or impossible with conventional immunohistochemical techniques (Figure 1, a and b). A reticular staining pattern was often noted, reflecting the deposition of PrP^{Sc} along cell borders, cellular processes, and vacuoles. PrP^{Sc} was detected in all three combinations of the polymorphism for methionine or valine at codon 129 of *PRNP*. The PET blot also detected PrP^{Sc} in nvCJD and in the different prion protein types as seen in Western blots according to Parchi et al.[6] CJD cases with a synaptic type of PrP^{Sc} deposition,[13] typically methionine homozygotes at codon 129 with PrP^{Sc} type 1, showed a delicate reticular staining throughout the cortex, frequently more pronounced in layers 5 and 6 and sometimes in layers 1–3 of the neocortex (Figure 1, d and e). Strong reticular staining was seen in the granular layer of the cerebellum, with some variation of staining in the molecular layer. The Purkinje cell layer was negative (Figure 1f). Valine homozygotes with PrP^{Sc} type 2 often showed, in immunohistochemistry, perineuronal staining in the neocortex and plaque-like deposits in the cerebellum. The PET blot in these cases showed granules, plaque-like and pericellular staining in the lower cortical layers, and plaque-like and granular staining in the granular cell layer and molecular layer of the cerebellum. In these cases granular and plaque-like deposits were often seen in the subcortical white matter of the cerebrum and cerebellum (Figure 1, g-i).

Valine homozygotes with protein type 1 are a rare but important subgroup of sporadic CJD (Figure 1, j-l). The patients are conspicuously young (we have seen three patients between 25 and 45 years of age), and the clinical course of progressive dementia is prolonged over 1 year. Typical EEG changes are not seen. Immunohistochemistry demonstrates almost no specific staining for PrP^{Sc}. As a consequence, the pathological diagnosis in these clinically atypical cases rests on the recognition of marked vacuolar changes in many areas of the brain, with little support from immunohistochemistry. With the PET blot technique, moderate to strong reticular staining is seen in the neocortex, basal ganglia, and cerebellum. This technique therefore must be considered as a means of unequivocally diagnosing this rare variant of sporadic CJD.

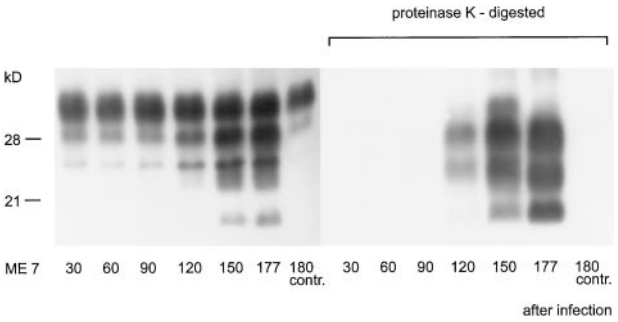

Figure 3. Western blot showing brain homogenates from C57/Bl6 mice 30, 60, 90, 120, 150, and 177 days after intracerebral infection with the ME7 scrapie strain. Each lane contains 1 mg of tissue; this is in the range of the amount of tissue used for a single PET blot. Proteinase K-resistant PrP^{Sc} is first observed 120 days after intracerebral infection. The polyclonal antisera K72, which was most sensitive in our experience, was used for immunodetection after blotting. contr., control.

To get a better estimate of the sensitivity of this new technique, it was compared to the histoblot technique, using a series of C57/Bl6 mice infected with the mouse-adapted scrapie strain ME7 (Figure 2). The PET blot first showed a delicate staining of the anterior part of the thalamus at day 30 after infection. Starting at day 60, PrP^{Sc} was detected in brainstem nuclei and the rostral cortex. The histoblot showed delicate PrP^{Sc} depositions in the frontal cortex, starting at day 60. Definite staining of the frontal cortex and hippocampus was noted at day 90. A detailed anatomical comparison in this series showed that in some areas a delicate staining was visible in PET blots 30 days before it was noted in histoblots, and definite and strong staining as a rule was also first visible in the PET blot 30 days before staining in the histoblot. Thus the PET blot is positive 30 days after infection, very early in the incubation time, and more than 140 days before the appearance of clinical signs 177 days after infection. In addition, a better anatomical resolution can be achieved with this new technique.

A quantitative comparison of the sensitivity of the PET blot with the Western blot technique was performed. Tissue lysates from one half of the brain of mice of the infection series used for the PET blot (Figure 2) were analyzed. Using an equivalent of 1 mg tissue per lane, we first detected PrP^{Sc} 120 days after infection with the ME7 strain (Figure 3). In comparison, with the PET blot technique PrP^{Sc} is detectable 30 days after intracerebral infection. Approximately 0.5–0.7 mg of tissue is required for one PET blot.

Figure 2. Comparison of the PET blot and histoblot techniques in the ME7 scrapie strain in C57/Bl6 mice 30, 60, 90, 120, 150, and 177 days after infection and in controls (c). The same monoclonal antibody directed against PrP (3B5), which has proved most sensitive, was used as the primary antibody for histoblots and PET blots. The two upper rows show sagittal sections in the PET blot technique, and the bottom rows show horizontal sections in the histoblot technique. Thirty days after infection there is well-delineated immunochemical staining in the anterior thalamus that increases in intensity and spreads 60 and 90 days after infection. After 60 days there is also beginning staining in the hippocampus and frontal cortex. The basal forebrain and upper brainstem nuclei are visible after 90 days. Strong staining of the cortex and inferior colliculus is observed after 120 days. After 150 and 177 days there is strong staining of most areas of the brain. Note that there is no staining of the cerebral and cerebellar white matter and the pyramidal layer of the hippocampus. The strongest staining signals are seen in the frontal cortex, hippocampus, inferior colliculus, and molecular layer of the cerebellum. Histoblots of a control mouse and a mouse 30 days after infection show faint background staining. A specific signal is first visible in the frontal cortex after 60 days. A faint hippocampal staining becomes visible after 90 days. Thus it appears that a faint but specific PrP^{Sc} staining can be observed in PET blots some 30 days before a signal is visible in histoblots. bg, basal ganglia; ce, cerebellum; cn, cerebral nuclei; co, cortex; hi, hippocampus; ic, inferior colliculus; ob, olfactory bulb; sc, superior colliculus; th, thalamus; c, control.

Discussion

We have developed a new technique for the detection of PrP^{Sc} in prion diseases of humans and animals that is largely based on a unique combination of preexisting methodologies. As we have shown, the PET blot can be used to identify PrP^{Sc} in all known variants of human sporadic CJD and may be used in the diagnosis of BSE and scrapie (data shown only for experimental scrapie in this publication). The PET blot is easy to perform as a routine procedure simultaneously with conventional immunohistochemistry.

The major advantages of the PET blot are its extremely high sensitivity (which, as we have shown, is superior to those of immunohistochemistry, Western blotting, and histoblotting), its satisfactory anatomical resolution, and its applicability to formalin-fixed and paraffin-embedded tissue. Thus archival neuropathological material can be studied. In addition, tissues can be decontaminated with formic acid after fixation in formalin, which makes tissue handling safer by several orders of magnitude.[10]

Although treatment of conventional histological sections for immunohistochemistry with proteinase K is possible, it is not routinely performed by many groups because this treatment has a very destructive effect on tissue sections on glass slides. Instead tissue pretreatment protocols such as hydrolytic autoclaving are used to destroy PrP^{C}, and the remainder is assumed to represent denatured PrP^{Sc}. As a result immunohistochemistry without preceding treatment with proteinase K detects an ill-defined mixture of PrP^{Sc} and PrP^{C} that may have escaped destruction by tissue pretreatment. The PET blot technique requires tissue pretreatment with high concentrations of proteinase K. As a consequence, only PrP^{Sc} is detected by this method. Conventional immunohistochemistry has a better microscopic resolution than the PET blot at the cellular and subcellular levels. However, the PET blot's morphological resolution is superior to that of the histoblot technique.

The technique will be useful in neuropathological diagnosis of CJD, particularly in brain biopsy diagnosis, detection of very small amounts of PrP^{Sc} in tissues as observed in lymphoid organs in nvCJD patients[4] and scrapie in sheep,[3] and to complement conventional immunohistochemistry, particularly in cases with equivocal results. This may be of great interest because PrP^{Sc} has been identified in the appendix of a nvCJD patient in the incubation time months before the onset of clinical signs.[5] As our infection series in mice has shown (Figure 2), the PET blot may facilitate the diagnosis of prion diseases in preclinical or early clinical stages in peripheral tissue.

In experimental prion research the PET blot may prove to show more clearly the anatomical and temporal distribution of PrP^{Sc} deposition in the brain and peripheral tissues. The technique may be used to reinvestigate unclear neurodegenerative diseases from neuropathological archival material. We envisage that PrP^{Sc} deposition will be detected in cases where it has so far been difficult or impossible to show in human neurodegenerative diseases that belong to the prion disease group, and we suggest that PET blot analysis will be instrumental in detecting PrP^{Sc} in BSE in early stages of the disease or even early in the incubation time in various tissues.

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