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Prion protein immunocytochemistry – UK five centre consensus report

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Prion protein immunocytochemistry – UK five centre consensus report

Creutzfeldt-Jakob disease (CJD) and other prion diseases are associated with the deposition of insoluble prion protein (PrP^{CJD}) in the central nervous system (CNS). Antibodies raised against PrP^{CJD} also react with its precursor protein, a soluble form of PrP (PrP^{C}), which is widely distributed in the normal CNS. This cross-reactivity has in the past raised doubts as to the specificity and diagnostic reliability of PrP immunolocalization, especially in familial cases which are atypical clinically and which lack characteristic pathology findings. Following an MRC-funded workshop which focused on this problem, a multicentre prospective study was set up to identify a reliable protocol for PrP^{CJD} immunocytochemistry. Five UK centres took part in this study and demonstrated consistent staining of plaques, vacuolar deposits in severe spongiform change, and perineuronal deposits using a variety of antibodies and enhancement procedures. A protocol using formic acid, guanidine thiocyanate, and hydrated autoclaving pre-treatment in conjunction with a monoclonal PrP^{CJD} antibody produced the clearest immunochemical results and is presented as the consensus UK recommendation for PrP^{CJD} immunocytochemical procedures.

Keywords: prion protein, immunocytochemistry, multicentre study, Creutzfeldt-Jakob disease, spongiform encephalopathy

Introduction

Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies are characterized by the deposition in the central nervous system (CNS) of an insoluble prion protein (PrP^{CJD}) [4, 28]. PrP^{CJD} is an aberrant form of a soluble precursor protein (PrP^{C}) which is encoded by a gene on chromosome 20 and is widely expressed in the normal CNS and other tissues but has a high rate of turnover and does not accumulate [26]. PrP^{CJD} has been detected in all the major human spongiform encephalopathies or prion diseases, including sporadic and familial CJD [24, 28], Gerstmann Sträussler Scheinker syndrome (GSS) [28] and fatal familial insomnia (FFI) [22, 28] as well as in iatrogenic cases, such as human growth hormone-related CJD [3, 29].

The factors which influence the appearance and accumulation of PrP^{CJD} are not wholly understood but the structure of the PrP gene is of central importance in familial prion diseases, including GSS and FFI [1]. Several different mutations, particularly at codons 178 and 200, have been described in the PrP gene of inherited cases, and homozygosity at codon 129 not only confers increased vulnerability to sporadic and iatrogenic prion diseases but also appears to influence the phenotype of mutations found at other codons [12, 27].

Disease-related PrP deposits are also found in animal prion diseases which include scrapie and bovine

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spongiform encephalopathy [14, 30]. Polyclonal antibodies have been raised to scrapie and hamster prion proteins, and both polyclonal and monoclonal antibodies directed against synthetic PrP peptides have become available more recently. Unfortunately none of these antibodies can clearly distinguish between PrP^{C} and PrP^{CJD} and dependable PrP immunocytochemistry should incorporate techniques which aim to eliminate PrP^{C} from tissue sections and enhance the demonstration of insoluble PrP^{CJD}.

The strategies which are currently available have been reviewed recently [2]. Briefly, the methods which are gaining general acceptance rely more on exploiting the likely quantitative differences between the two isoforms by exposing tissue sections to high dilution anti-PrP antibody. Many immunocytochemical studies of human prion diseases have now been published but some difficulties in interpretation remain to be resolved [7, 15–17, 19–21, 23, 25]. While there is no dispute concerning the immunolocalization of PrP^{CJD} in the amyloid plaques which occur in some cases of prion disease, particularly in the cerebellum, agreement regarding other PrP^{CJD}-positive deposits has been less than unanimous [2]. There is a range of patterns of pathology, both in topography and quantity of spongiform change, gliosis and plaque formation, which may or may not be accompanied by different patterns of PrP^{CJD} deposition. A further problem relates to so-called atypical cases of prion disease in which PrP gene mutations are associated with dementing illnesses but not with characteristic spongiform or plaque pathology [8]: in such cases no consensus has been achieved with respect to PrP^{CJD} immunolocalization and reliable protocols would be particularly important in their diagnostic validation.

Accurate identification of PrP^{CJD} in tissue sections is of interest not only because of the diagnostic implications but also because of current theories which identify PrP^{CJD} as a most significant part of the infective agent, if not its entirety [11, 26]. Immunocytochemistry may lend itself to studies of PrP^{CJD} mapping and spread in the CNS [2], and possibly in other organs and tissues such as the pituitary which have been identified as sources of infection [5].

In 1993 the Medical Research Council was aware of the need to achieve an agreed protocol for PrP immunocytochemistry which would lead to reproducible results and a consensus view on the interpretation of findings. First, a one day Workshop was arranged to draw together scientists and technical staff from eight centres engaged in immunocytochemical study of human and animal prion diseases. Subsequently, a five centre trial of PrP immunocytochemical methodology was set up with the aim of standardizing protocols and agreeing the interpretation of results, particularly with regard to atypical prion dementias. Each of the five centres had some experience of implementing PrP immunocytochemistry in human cases of prion disease, varying from occasional to almost daily use.

The achievement of UK consensus for PrP^{CJD} immunolocalization, which is an immunocytochemical procedure more than usually beset with problems of sensitivity, specificity and false positives, could be an important contribution for comparison of future studies in this field.

Materials and methods

Five participating centres were identified (Nottingham, Belfast, Edinburgh, and St Mary's Hospital and the Institute of Psychiatry, both in London). The co-ordinating centre was Edinburgh. Each centre was originally invited to submit unstained sections from an individual case of interest which were to be sent for PrP staining to all five centres, but it was rapidly agreed that the final selection of cases should include three cases from Edinburgh and two cases from the Institute of Psychiatry since these were the centres with the widest range of material. The cases were selected to include a range of pathology as revealed by routine haematoxylin and eosin staining and preliminary PrP immunostaining, and comprised four cases of sporadic CJD and one case of atypical prion disease submitted from London [8]. The four cases of sporadic CJD did not have PrP mutations and showed varying patterns of pathology. The atypical prion disease case was a member of a kindred with a 144 base pair PrP gene insertion, with a long history of personality disorder preceding dementia but with no evidence of spongiform change (case VII 63 in reference 9). From each case three blocks were chosen including cerebral and cerebellar cortex and basal ganglia. CJD material was exposed to formic acid for 1 h during processing of tissue blocks and this is thought to be effective for decontamination of formalin fixed tissue [6].

The sets of unstained sections were collated in Edinburgh, coded by an independent member of staff and distributed to the five centres where staining was to be

Table 1. PrP immunostaining protocols used in the five centres

	Antibody (dilution)	*Pre-treatment*	*Visualizing agent*
Centre 1	SP40 (1 in 1000 o/n at 4°C)	98% formic acid (3 min) and 4 M guanidine thiocyanate (2 h)	Diaminobenzidene
Centre 2	SP40 (1 in 3600 o/n at 4°C)	98% formic acid (10 min)	Aminoethyl carbazol
Centre 3	1A8 (1 in 1800 o/n at 4°C)	80% formic acid (8 min)	Diaminobenzidene
Centre 4	1A8 (1 in 2000 o/n at 4°C)	96% formic acid (5 min)	Diaminobenzidene
Centre 5	1A8 (1 in 1800 o/n) KG9 (1 in 200 o/n) (both at room temperature)	96% formic acid (5 min) and 4 M guanidine thiocyanate (2 h) with or without prior hydrated autoclaving	Diaminobenzidene

o/n, Overnight exposure.

Table 2. PrP antibodies used in the five centre study

Antibody	*Clonality*	*Antigen*	*Source*
1A8	Polyclonal	Denatured scrapie fibrils in ME7 mouse strain	Dr J. Hope (Edinburgh)
SP40	Polyclonal	Synthetic peptide based on sheep PrP gene sequence residues 219–232	Professor B. Anderton (London)
KG9	Monoclonal	Recombinant protein based on shared bovine/human PrP sequence	Dr C. Birkitt (Compton)

undertaken. Each centre employed the antibody and the protocol which had been in use in that centre up to that time (Table 1). Avidin biotin amplification was used in each centre. The stained slides were then returned from individual centres to Edinburgh whence the full coded set of slides was circulated for assessment of staining to each centre in turn. The full set was finally returned to Edinburgh where the extent of pathology findings in the five cases was also assessed with routine haematoxylin and eosin staining.

To collate the results, written comments were elicited from each centre for preliminary analysis in Edinburgh. In addition to identifying the antibody and the pre-treatment and visualizing protocols in use in each centre and detailed in Table 1, each centre was asked to: (a) comment specifically on the pattern of PrP staining and whether this was believed to be specific or otherwise; (b) provide an assessment of artefactual and background staining; (c) comment on individual antibody and protocol performance in the slides from each coded centre. A draft summary of the results and conclusions was circulated for comment before the final consensus report was agreed between all centres for submission to the Medical Research Council.

Details of the antibodies used in the different centres are shown in Table 2. The specificity of these antibodies had been checked in preliminary studies by use of antibody pre-absorbed against the specific antigen [2, 17]. In all five centres the pre-treatment of tissue sections with formic acid was in routine use prior to this study. The omission of such a step produced unacceptable levels of background staining, since in these circumstances it was necessary to drop the dilution of antibody very considerably to achieve visualization of disease-related structures. Negative controls included sections not exposed to the primary antibodies. The use of more extensive controls, including other dementias and age matched non-demented subjects, has been described and validated previously for these PrP antibodies and protocols [2, 17]. These controls have been consistently

Table 3. Five centre PrP study. Pathology findings as revealed by haematoxylin and eosin staining together with PrP^{CJD} immunolocalization pattern in five selected cases of prion disease

	Case 1	*Case 2*	*Case 3*	*Case 4*	*Case 5*
(A) *Cerebral cortex*					
Spongiform change	+	++	++	++	No significant pathology
PrP^{CJD} positivity	Plaques++ (Perineuronal)	Vacuolar	Vacuolar	Plaques ± (Perineuronal)	PrP-negative
(B) *Basal ganglia*					
Spongiform change	++	±	+	++	No significant pathology
PrP^{CJD} positivity	Plaques++	Plaques+	(Astrocytes)	Plaques+	PrP-negative
(C) *Cerebellar cortex*					
Spongiform change	No significant pathology	No significant pathology	+	+Kuru-type plaques	Atrophy
PrP^{CJD} positivity	Plaques++	Plaques ±	PrP-negative	Plaques++	Atypical vertical linear deposits in molecular layer

Spongiform change (± very slight & focal; +microcystic; ++severe & confluent). PrP-positive plaques (± occasional; +focally numerous; ++widespread and numerous, present in all cerebellar layers). Staining patterns which are bracketed were agreed by less than five centres (see Results).

negative for all the antibodies used in the multicentre study.

Results

Routine pathology

The test cases were deliberately selected for differing pathological appearances and topographical distribution of spongiform change and plaques. These differences are summarized in Table 3 and underline the between-case variability in prion diseases. Case 4 was the only case in which numerous kuru-type plaques were readily identified by routine staining in the cerebellum. Cases 1–4 all showed spongiform change either in the cerebral or cerebellar cortex, or in the basal ganglia, and sometimes in all three. This took the form of small individual vacuoles diffusely scattered through the parenchyma (Figure 1), as in some areas of the cerebral cortex and in the molecular layer of the cerebellum, or in larger irregularly confluent vacuoles present in the grey matter of the cerebral cortex (Figure 2) or of basal ganglia.

Neuronal loss and astrocytosis may also be found in CJD and were present in varying degree in cases 1–4. In case 5, no spongiform change or other characteristic pathology was detected in the cerebral cortex or in the basal ganglia; the cerebellar cortex showed some atrophic changes with loss of granular neurons and astrocytosis, but without evidence of spongiform change in the molecular layer.

PrP immunolocalization

PrP-positive features were identified in all five cases in at least one block by each of the five centres. Kuru-type spherical plaques first seen indistinctly in haematoxylin and eosin stained sections of the cerebellum of case 4 were readily confirmed as PrP-positive (Figure 3) and plaques were first revealed by immunocytochemistry in cases 1 and 2 (Table 3). All five centres identified these PrP positive plaques as consistently stainable features which varied in number and distribution between cases. However, PrP-positive plaques were not revealed in case 3. In the experience (unpublished) of the Edinburgh CJD Surveillance Unit, plaques are found in about 15% of cases of sporadic CJD. PrP-positive plaques were present in the molecular and granular layers of the cerebellum (Figure 4) as well as in the white matter. They may also be present in the cerebral cortex and in the basal ganglia and this was well demonstrated in case 1 as agreed by all five centres (Table 4).

In case 5 unusual linear deposits were conspicuous in the molecular layer of the cerebellum and this pattern

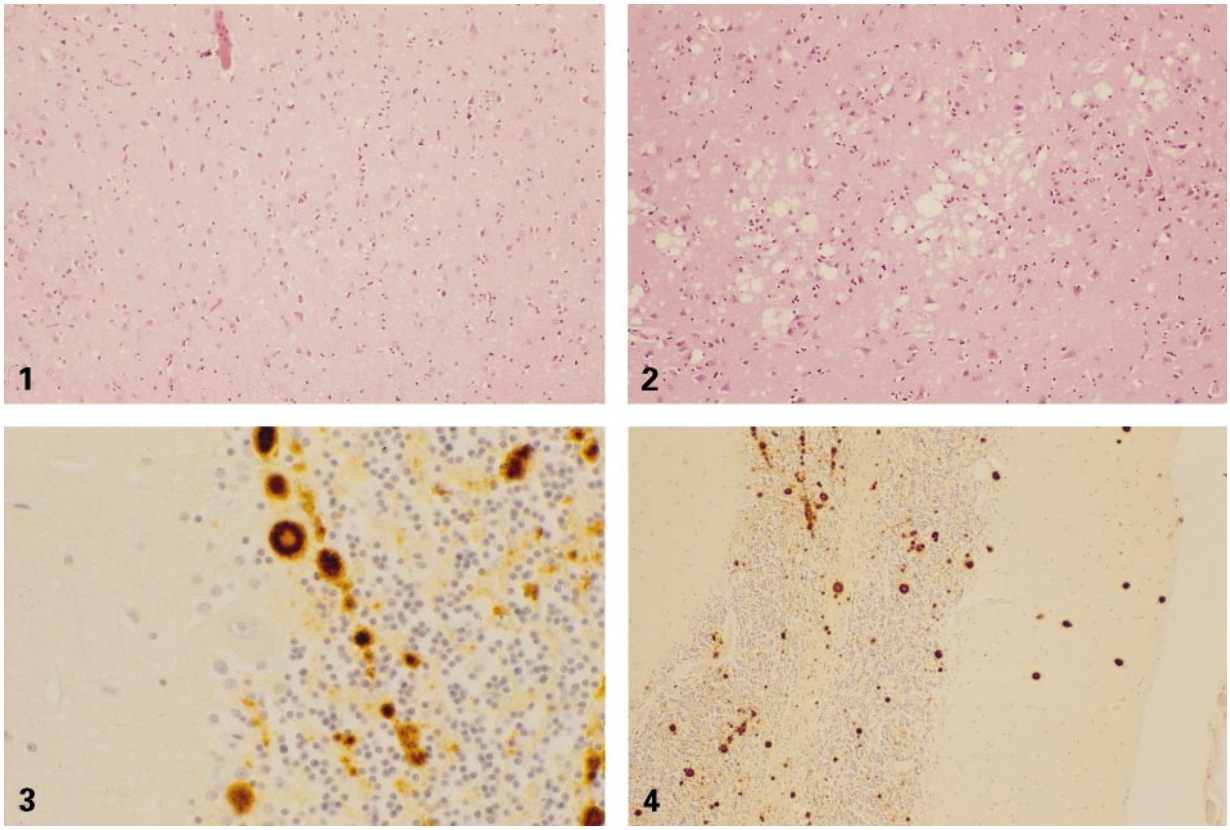

Figure 1. Cerebral cortex of case 1 showing diffusely scattered small vacuoles in the neuropil. Haematoxylin and eosin. ×100.

Figure 2. Cerebral cortex from case 2 showing more severe confluent spongiform change in the neuropil. Haematoxylin and eosin. ×100.

Figure 3. Section of cerebellum from case 4 showing plaques stained immunocytochemically with antibody to KG9 (see Table 2). The Purkinje cells lying between the darkly staining plaques are negative. ×400.

Figure 4. Cerebellum from case 1 stained with the anti-PrP antibody SP40. Positive plaques are revealed predominantly in the granular layer with occasional plaques in the molecular layer and in the thin strip of white matter which traverses the centre of this narrow folium. ×100.

was identified by all five centres (Figures 5 and 6). PrP positivity was not present elsewhere in this case and the Purkinje cells were negative.

PrP-positive plaques are characteristically spherical and may have a pale staining centre with a spiculated radial dark-staining periphery – the so-called classic kuru plaque.

PrP-positive deposits of less regular arrangement were also identified in relation to severe confluent spongiform change in the cerebral cortex (Figure 7). All five centres noted this staining pattern. No consistent PrP positivity was observed in grey matter in which spongiform change was of a microcystic pattern.

Two of the five centres also drew attention to occasional evidence of apparent perineuronal PrP deposition in the cerebral cortex of cases 1 and 4 (Figure 8). These two centres also commented on punctate PrP staining in the neuropil and slight positivity of glial cells with both monoclonal and polyclonal antibodies (case 3) (Figure 9). The remaining centres did not comment on these last features. Occasional and inconsistent staining of blood vessels was seen with the antibody KG9 as used in centre 5. Not all the blocks in any one case showed this phenonemon and subarachnoid vessels were sometimes affected as well as central white matter vessels. There was no evidence in these five cases of the diffuse synaptic pattern of positivity which has been described in the cerebellar molecular layer [19].

In summary, all five centres agreed on the specificity of PrP staining in plaques. All five centres also agreed on

Table 4. Consensus protocol for PrP^{CJD} immunocytochemistry

1	5 μm sections floated on to Vectabond-coated slides
2	Sections to water
3	Picric acid 15 min
4	Water
5	3% hydrogen peroxide 30 min
6	Water
7	Hydrated autoclaving (121°C for 10 min in distilled water)
8	Water
9	96% formic acid for 5 min
10	Water
11	4 M guanidine thiocyanate for 2 h at 4°C
12	Water then Tris buffered saline
13	Blocking serum for 20 min
14	Exposure to primary PrP antibody (see Table 2)
15	Tris buffered saline
16	Exposure to secondary antibody
17	Tris buffered saline
18	ABC kit
19	Tris buffered saline
20	Visualizing agent
21	Water
22	Haematoxylin counterstain
23	Dehydration, clearing and mount in Pertex
24	Dried, mounted slides may be decontaminated again by immersion in 96% formic acid for 5 min before labelling. This is regarded as a useful additional precaution as the slides leave a possibly contaminated laboratory

the curious staining pattern in the molecular layer of the atypical prion case (case 5). Four of the five centres agreed that the diffuse PrP deposits seen in relation to severe cerebral cortical spongiform change were also specific. Other features such as perineuronal and glial PrP staining merited comment from only two of the five centres.

Variability of PrP immunostaining results: antibodies and protocol

Despite the different antibody and pre-treatment combinations in the five centres, inspection of the five stainings of each of the 15 blocks showed the same pattern of chromagen deposition, although the intensity of staining varied between the different centres. All the centres agreed that the same structures, including plaques, or vacuolar deposits in association with spongiform change, or deposits associated with neurons, were revealed by the different staining protocols adopted by the five centres but they also commented that certain protocols from one or two of the centres, employing one rather than another antibody, consistently revealed more of the stainable features than did other protocols. Four of the five centres thought that the monoclonal antibody, KG9, produced the most intense and widespread staining of plaque and vacuolar deposits and of neuronal associated staining. One centre believed that SP40 gave the best results. All centres were agreed that SP40 gave very good results preferable to those produced with 1A8. All five centres concurred that, although formic acid was an essential pre-treatment, based initially on guidance in the literature and then on experience of all five laboratories, as discussed at the workshop (and reviewed in [2]), the addition of autoclaving, as described previously, was a very useful additional enhancing step which produced darker and better defined deposits of all the three types specified above (Figures 5 and 6). If one feature was enhanced by a particular antibody pre-treatment, then all were similarly affected. The value of guanidine thiocyanate as a further 2 h pre-treatment used in centre 5 was questioned by the remaining four centres who did not detect any additional benefit in the intensity or specificity of staining and, in the opinion of one centre, it caused slightly higher background staining although allowing a greater dilution of the primary antibodies. However, based on judgement of the staining patterns identified in the slide circulation, the participating centres agreed to recommend the protocol used in centre 5 as the best in this study for demonstrating plaques, vacuolar staining and neuronal associated deposits. This protocol is shown in Table 4.

All the centres observed occasional staining of vessels with the monoclonal antibody KG9 as used by centre 5. Apparently positive vessels were particularly noticeable in the subarachnoid space and focally in the central white matter (Figure 10) but were present in no more than one block from each individual case. This has also been observed when control sections of normal brain and other forms of neurodegenerative diseases are stained with this antibody.

Discussion

This multicentre study has shown that the staining patterns resulting from different PrP immunocytochemical protocols are consistent and reproducible between laboratories, thus validating the results. We have shown previously that positivity for these antibodies is not found in other dementias such as Alzheimer's disease, or in

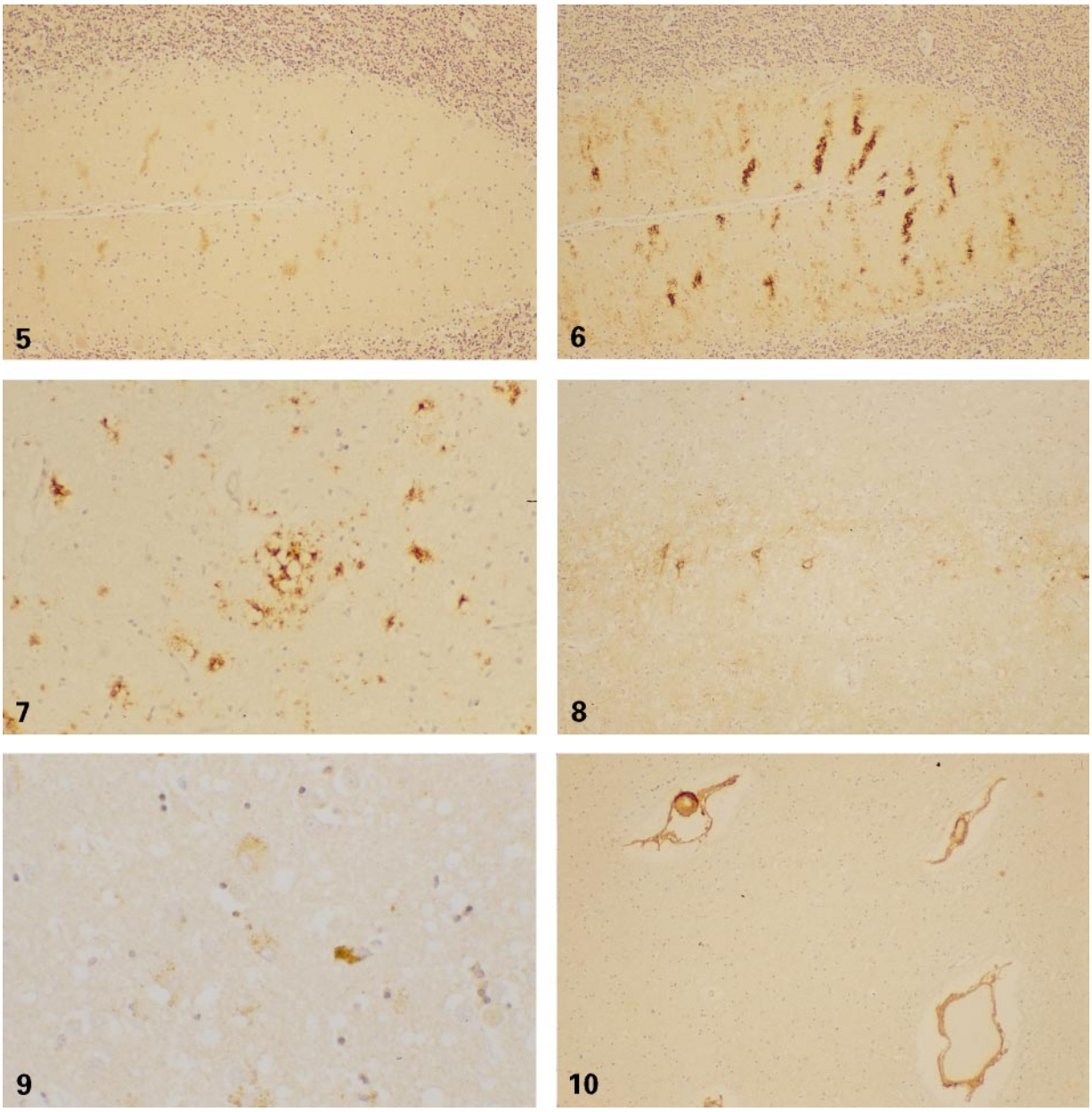

Figure 5. Cerebellar folia from a patient with atypical prion disease (case 5) exposed to the antibody KG9 without autoclave pretreatment. Faint linear positivity is noted in the molecular layer. × 100.

Figure 6. Section from the same case shown in Figure 5. In this section the PrP immunocytochemistry using the antibody KG9 has included a hydrated autoclave pretreatment. This pretreatment step represents the only difference between the sections shown in Figures 5 and 6 and demonstrates the enhancement which can be achieved by prior autoclaving. × 100.

Figure 7. Cerebral cortex from case 2 showing severe spongiform change associated with irregular deposits of prion protein visualized following exposure to the antibody KG9. × 200.

Figure 8. Section of cerebral cortex from case 4 showing laminar staining of neuronal membranes when exposed to anti-PrP antibody SP40. × 200.

Figure 9. Section of basal ganglia from case 3 showing spongiform change and small deposits of PrP immunopositivity with antibody KG9. In addition some astrocytes show granular cytoplasmic positivity such as the cell in the centre of the field. × 400.

Figure 10. Apparently artefactual PrP immunopositivity (KG9) in the walls of blood vessels in the central white matter of case 2. × 40.

non-demented age-matched controls [2, 17]. PrP-positive features such as plaques and perivacuolar deposits in areas of confluent spongiform change are broadly agreed. If these two patterns of PrP immunostaining are used as a basis of case judgement, it was clear from the present study that the use of different antibodies, both polyclonal and monoclonal, produced broadly similar staining patterns. It is also clear that employment of different protocols in different laboratories all produced similar staining patterns in the different cases and it is important to note that a formic acid pre-treatment step was used in all cases. Formic acid has a well established role in enhancing immunostaining for different forms of amyloid and other proteins in tissue sections [10, 18]. It was agreed that hydrated autoclaving was a significant further enhancing step but not all laboratories have ready access to the necessary equipment and it is reassuring to note that formic acid alone produces broadly similar results. What is not clear at this stage is whether autoclaving reveals small quantities of PrP in cases which would otherwise be negative and further study is required in this area. It is possible that pre-treatment with guanidine thiocyanate might be excluded with no great detriment to the protocol.

Examination of these five cases underlines the topographical variation in pathology and in PrP positivity from case to case of prion disease. Examination of autopsy cases allows the possibility of examining a minimum of several blocks from an individual case and highlights the difficulty of making a diagnosis on a single block of tissue such as a biopsy specimen. Examination of the PrP staining results in case 3 and in case 5 underlines this point (Table 4). Comparison of the results of routine staining and PrP immunolocalization (Tables 1 and 4) also shows that superficially similar cases (cases 1 and 2) may show quite different patterns of PrP deposition. The fact that independent examination in each of the five centres reproduced the same PrP staining patterns in this pair of cases generates considerable confidence that this is a reliable technique for detection of disease related cases.

In summary, the conclusion of this study is that PrP staining patterns may vary in intensity with different antibodies and protocols but that they are nevertheless very consistent between laboratories, and are endorsed as specific to prion diseases. Kuru-type plaques and deposits associated with confluent spongiform change are consistently PrP-positive. It was valuable for five separate laboratories to have the opportunity of examining a so-called atypical case with a PrP gene insertion and no characteristic pathology and for each to have demonstrated the presence of irregular linear PrP deposits in the cerebellar molecular layer. The Purkinje cell staining which was reported previously in this case [9] could not be confirmed. While some debate exists between the five centres as to the specificity of PrP localization in astrocytes it is likely that this issue will be resolved with greater experience of PrP immunolocalization. It was interesting to note that centre 1 submitted some sections of mouse brain in which prion disease was present and that in these sections glial staining was conspicuous in addition to positive plaques. It may be that astrocyte staining, which has been described previously in murine scrapie [13], is more noticeable in transmitted animal cases than in the human diseases. The general conclusion from all five centres is that staining of blood vessels is an artefactual feature which does not interfere with interpretation of specific results.

The consensus optimal protocol for PrP immunolocalization which has emerged from this study is outlined in Table 4. Pre-treatment with formic acid is regarded as mandatory. The additional step of hydrated autoclaving is desirable if there is ready access to an autoclave or even to a pressure cooker within the laboratory. It is reassuring to note that all the antibodies used in this study produced staining of the same pattern although results appeared to be optimal with a monoclonal antibody raised to a recombinant protein.

The establishment of agreed and validated protocols for PrP immunolocalization provides the way forward for diagnostic confirmation of PrP deposition in typical and atypical cases. The apparent discordance between classical pathology findings and PrP deposition in some areas of the CNS such as the molecular layer of the cerebellar cortex awaits explanation. This has been noted previously [2] and is confirmed in the present study.

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Addendum

Because autoclaves are not generally available within histopathology laboratories, the CJD Surveillance Unit has experimented since the multicentre study with microwave substitution. We have found that the following step may be substituted for step 7 in Table 4.

Alternative step 7

(a) Microwave at full power for 5 min in distilled water.
(b) Allow to cool.
(c) Repeat step (a) above.
(d) Allow to cool and proceed with step 8 of Table 4.

This alternative procedure enhances PrP^{CJD} immunopositivity almost as effectively as autoclaving.

The protocol listed in Table 4 was the one used for the demonstration of PrP^{CJD} immunoreactivity in the new variant cases of CJD which were reported recently (Will *et al.* A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* 1996; **347:** 921–6).

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