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Human prion diseases: from antibody screening to a standardized fast immunodiagnosis using automation

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Demonstration of pathological prion protein accumulation in the central nervous system is required to establish the diagnosis of transmissible subacute encephalopathies. In humans, this is frequently achieved using prion protein immunohistochemistry in paraffin-embedded tissue, a technique that requires multiple epitope retrieval and denaturing pretreatments. In addition to being time-consuming, this procedure induces tissue alterations that preclude accurate morphological examination. The aim of this study was to simplify prion protein immunohistochemistry procedure in human tissue, together with increased sensitivity and specificity. We screened a panel of 50 monoclonal antibodies produced using various immunogens (human and ovine recombinant prion protein, prion protein peptides, denatured scrapie-associated fibrils from 263K-infected Syrian hamsters) and directed against different epitopes along the human prion protein sequence. A panel of different forms of genetic, infectious and sporadic transmissible subacute encephalopathies was assessed. The monoclonal 12F10 antibody provided a high specificity and fast immunodiagnosis with very limited denaturing pretreatments. A standardized and reliable fast immunostaining procedure was established using an automated diagnostic system (Nexes, Ventana Medical Systems) and allowed prion protein detection in the central nervous system and in tonsil biopsies. It was evaluated in a series of 300 patients with a suspected diagnosis of transmissible subacute encephalopathies and showed high sensitivity and specificity.
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The accumulation of a specific protease-resistant isoform (PrP^{sc}) of the host-encoded prion protein (PrP^{c}) characterizes human transmissible spongiform encephalopathies. Identifying PrP^{sc} in the central nervous tissue of suspected patients is a critical step for the diagnosis of definite human transmissible subacute encephalopathies.[1,2] This is frequently achieved using PrP immunohistochemistry on formalin-fixed tissues.

Retrieval of antigens and denaturing pretreatments is required for detecting PrP^{sc} by immunohistochemical procedures in sections of formalin-fixed and paraffin-embedded tissues. Formic acid[3] and enzymatic digestion with proteinase K[4] or pepsin[5] have been used at first. Denaturing treatments using guanidine thiocyanate[6] and heating by hydrolytic autoclaving[7] or microwave irradiation[8] and hydrated autoclaving[9] have also been developed. Various combinations of these methods have been successfully tested.[10,11] Two multicentric, prospective studies have assessed several antibodies, including the commercially available and widely used 3F4[12] antibody in combination with enhancement procedures. Regardless of the antibody in use, they have led to the same reliable standardized pretreatment protocol for PrP^{sc} immunohistochemistry: hydrated autoclaving (121°C for 10 min), followed by formic acid (96% for 5 min) and guanidine thiocyanate (4 M, 4°C for 2 h).[13] However, the most sensitive procedure needs an additional antigen-retrieving treatment (ie, proteinase K digestion),[14] and these complex pretreatments are time-consuming and induce tissue alterations that preclude fine morphological analysis.

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Using a large panel of anti-PrP monoclonal antibodies produced with various immunogens, we have developed a fast, reliable and automated procedure for PrP^{sc} immunohistochemistry in human samples, which enables (1) a high sensitivity and specificity for the diagnosis of human transmissible subacute encephalopathies and (2) a fine preservation of morphological information thanks to a simplified antigen-retrieving procedure.

Materials and methods

Antibody Screening Using a Four-Step Strategy

Step 1: Screening of 50 antibodies on four patients using a combination of four pretreatments
Our strategy was to study a panel of 50 monoclonal antibodies on four patients using a combination of four pretreatments. Four dilutions of primary antibodies (1/100, 1/500, 1/1000, 1/2000) were tested. The staining was compared to that obtained using the 3F4 antibody (Figure 1, Tables 1 and 2).

Step 2: Reducing the pretreatments in the six selected antibodies
The highly efficient antibodies identified in step 1 were used in different simplified pretreatment procedures.

Step 3: Automation of the procedure using the six selected antibodies on 26 patients
These antibodies were checked for their efficiency at 37°C using an automated diagnostic system for immunohistochemistry (Nexes, Ventana Medical Systems, Illkirch, France) on 26 patients. The results were compared to those obtained with 3F4 antibody using the manual and four pretreatment procedures. The highest sensitivity was obtained with the 12F10 antibody.

Step 4: Validation of the automated procedure using an additional series of 25 sporadic Creutzfeldt–Jakob disease patients
Together with the 26 patients used in step 3, sections from a total of 51 patients were used to compare the 12F10 automated procedure to the manual procedure, with the reference 3F4 antibody.

Patients and Samples

The panel of 51 patients, which included 46 patients with neuropathologically confirmed transmissible subacute encephalopathies, is described in Table 1. Paraffin-embedded blocks of 10% formalin-fixed brains were sampled from the frontal isocortex and the cerebellar vermis. Serial sections cut at 7 μm on superfrost-plus slides were dried at 56°C for 24 h before immunohistochemical procedures.

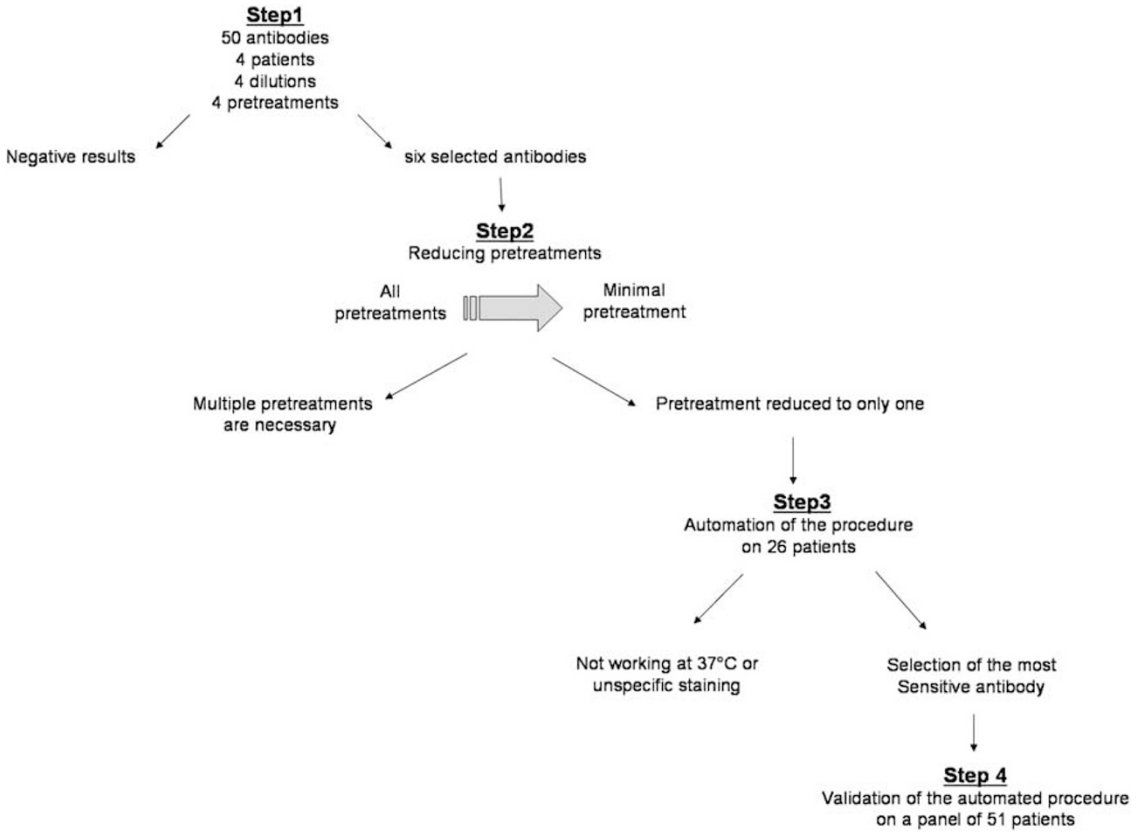

Figure 1 Strategy used for antibody screening.

Table 1 Patient characteristics

Step 1 and step 2 (4 patients)	*Step 3 (26 patients)*	*Step 4 (51 patients)*
sCJD MM1 ($n=1$)	sCJD MM1 ($n=3$)	sCJD MM1 ($n=20$)
sCJD MV1 ($n=1$)	sCJD MV1 ($n=3$)	sCJD MV1 ($n=8$)
sCJD VV2 ($n=1$)	sCJD MV2 ($n=3$)	sCJD MV2 ($n=3$)
AD ($n=1$)	sCJD VV2 ($n=3$)	sCJD VV2 ($n=6$)
	vCJD ($n=3$)	vCJD ($n=3$)
	GSS P102L ($n=1$)	GSS P102L ($n=1$)
	FFI ($n=1$)	FFI ($n=1$)
	fCJD E200K ($n=1$)	fCJD E200K ($n=1$)
	iCJD ($n=3$)	iCJD ($n=3$)
	AD ($n=3$)	AD ($n=3$)
	NND ($n=2$)	NND ($n=2$)

AD, Alzheimer disease patient; fCJD, inherited Creutzfeldt–Jakob disease with the E200K mutation; FFI, familial fatal insomnia with the D178N-129M mutation; GSS, Gerstmann–Sträussler–Scheinker syndrome with the P102L mutation; iCJD, iatrogenic CJD after extractive growth hormone treatment; MM1, methionine homozygosity at codon 129 of PRNP and a PrP^{sc} type 1; MV1, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 1; MV2, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 2A; NND, patients who died from non-neurological diseases; sCJD, sporadic Creutzfeldt–Jakob disease; vCJD, variant Creutzfeldt–Jakob disease; VV2, valine homozygosity at the codon 129 and a PrP^{sc} type 2A.

Immunohistochemical Procedures

Pretreatments

A combination of four pretreatments was used: hydrated autoclaving in distilled water (25 min), formic acid (5 min, 99%, 24°C), guanidine thiocyanate (2 h, 4 M, 4°C), and proteinase K (8 min, 10 μg/ml, 24°C).

Anti-PrP antibodies

Antibodies were produced in $PrP^{0/0}$ mice using different immunogens (human and ovine recombinant PrP, PrP peptides, denatured scrapie-associated fibrils from 263K-infected Syrian hamsters), and have been described elsewhere.[15,16] They recognized different epitopes along the PrP sequence (Table 2). Monoclonal antibodies were purified using either caprylic acid precipitation[17] or protein A affinity chromatography. Epitope mapping for identifying linear epitopes possibly recognized by monoclonal antibodies was performed as previously described.[16] 3F4 antibody (ref. 9620) was produced and commercialized by Signet Laboratories (MA, USA).

Manual procedure

After a saturation step using 10% goat serum for 30 min, a 3% H_2O_2 blockade of endogenous peroxidases and different combinations of pretreatments, the primary antibody was applied overnight at room temperature. A standard streptavidin–biotin peroxidase kit (ref. K5001) with diaminobenzidine substrate (Dako, Trappes, France) was used for single immunolabelling according to the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated and mounted.

Table 2 Antibodies screened

Antibody	*Isotype*	*Epitope*	*Immunogen*
3F4	IgG2a	109–112	PrP^{sc} hamster 27–30
Pri-101	IgG2a	23–33	Human PrP^{c}, peptide 23–33
Pri-102	IgG2a	23–33	Human PrP^{c}, peptide 23–33
Pri-103	IgG2bk	23–33	Human PrP^{c}, peptide 23–33
Pri-108	IgG1	23–33	Human PrP^{c}, peptide 23–33
Pri-151	IgG1	23–33	Human PrP^{c}, peptide 23–33
Pri-152	IgG1k	23–33	Human PrP^{c}, peptide 23–33
Pri-153	IgG1	23–33	Human PrP^{c}, peptide 23–33
Pri-154	IgG1k	23–33	Human PrP^{c}, peptide 23–33
Pri-157	IgG2a	23–33	Human PrP^{c}, peptide 23–33
Pri-303	IgG2ak	106–126	Human PrP^{c}, peptide 106–126
Pri-304	IgG1k	111–117*	Human PrP^{c}, peptide 106–126
Pri-305	IgG2ak	106–126	Human PrP^{c}, peptide 106–126
Pri-307	IgG2ak	106–126	Human PrP^{c}, peptide 106–126
Pri-308	IgG1k	111–118*	Human PrP^{c}, peptide 106–126
Pri-310	IgG1k	106–126	Human PrP^{c}, peptide 106–126
Pri-313	IgG1k	106–126	Human PrP^{c}, peptide 106–126
Pri-622	IgG2ak	161–179	Human PrP^{c}, peptide 161–179
Pri-901	IgG1	213–233	Human PrP^{c}, peptide 213–233
Pri-904	IgA	213–233	Human PrP^{c}, peptide 213–233
Pri-907	IgG1	213–233	Human PrP^{c}, peptide 213–233
Pri-908	IgG1	213–233	Human PrP^{c}, peptide 213–233
Pri-909	IgG1	213–233	Human PrP^{c}, peptide 213–233
Pri-917	IgG1	216–221*	Human PrP^{c}, peptide 213–233
14D3	IgG1k	37–53	Human recombinant PrP
4F2	IgG2bk	59–89*	Human recombinant PrP
3B5	IgG2ak	59–89*	Human recombinant PrP
8G8	IgG2ak	97–102*	Human recombinant PrP
12F10	IgG2ak	144–152*	Human recombinant PrP
11C6	IgG2ak	D/U	Human recombinant PrP
SAF15	IgG3k	79–92	Denatured PrP^{sc} 263K
SAF31	IgG2bk	79–92	Denatured PrP^{sc} 263K
SAF32	IgG2bk	79–92	Denatured PrP^{sc} 263K
SAF33	IgG2bk	79–92	Denatured PrP^{sc} 263K
SAF34	IgG2ak	79–92	Denatured PrP^{sc} 263K
SAF35	IgG2bk	79–92	Denatured PrP^{sc} 263K
SAF37	IgG2bk	79–92	Denatured PrP^{sc} 263K
SAF53	IgG2ak	143–153*	Denatured PrP^{sc} 263K
SAF54	IgG2bk	157–161*	Denatured PrP^{sc} 263K
SAF60	IgG2bk	157–161*	Denatured PrP^{sc} 263K
SAF61	IgG2ak	142–153*	Denatured PrP^{sc} 263K
SAF66	IgG2a	142–160*	Denatured PrP^{sc} 263K
SAF69	IgG2bk	157–161*	Denatured PrP^{sc} 263K
SAF70	IgG2bk	156–162*	Denatured PrP^{sc} 263K
SAF75	IgG2ak	144–149*	Denatured PrP^{sc} 263K
SAF76	IgG2ak	144–149*	Denatured PrP^{sc} 263K
SAF83	IgG1k	D/U	Denatured PrP^{sc} 263K
SAF84	IgG2bk	161–170*	Denatured PrP^{sc} 263K
BAR224	IgG2a	141–151	Ovine recombinant PrP
BAR226	IgG2a	D/U	Ovine recombinant PrP
BAR233	IgG2a	141–152*	Ovine recombinant PrP

D/U, discontinuous/unidentified; PrP^{c}, host-encoded prion protein; PrP^{sc}, protease-resistant isoform of prion protein.
*Antibodies recognizing a linear epitope determined by a pepscan method (see Materials and methods).

Automated procedure

A saturation step using 3% BSA for 20 min and hydrated autoclaving pretreatment was applied. The NexES IHC automated diagnostic system for immunohistochemistry and the iVIEW DAB Paraffin kit (ref. 760-091) were used according to the manufacturer's instructions (Ventana Medical Systems). The blocking of endogenous peroxidases, primary antibody incubation (32 min), DAB/copper

Table 3 Search for the simplified pretreatments for each optimal adjusted dilution (manual procedures)

Antibody	*Dilution (μg/ml)*	*Different pretreatment procedures*							
		None	*HA*	*FA*	*PK*	*HA+FA*	*HA+GT*	*HA+FA+GT*	*HA+FA+GT+PK*
3F4	1/500 (2.00)	−	−	−	−	−	−	+	++
12F10	1/2000 (0.85)	+	++	+	+	++	++	++	++
4F2	1/500 (2.00)	−	++	−	−	++	++	++	++
3B5	1/2000 (0.95)	+	++	+	+	++	++	++	++
SAF54	1/500 (0.80)	+	++	+	+	++	++	++	++
SAF34	1/1000 (1.10)	+	++	−	+	++	++	++	++
BAR233	1/500 (2.00)	−	++	−	−	++	++	++	++

FA, formic acid; GT, guanidine thiocyanate; HA, hydrated autoclaving; PK, proteinase K.
The level of positive immunostaining rated on a three-grade scale: −, no staining; +, positive staining; ++, strong positive staining.

revelation and section counterstaining with haematoxylin were performed at 37°C working temperature. Slides were dehydrated and mounted.

PrP Patterns

For assessing the pattern of immunolabelling with anti-PrP antibodies on brain samples, we followed criteria derived from those used previously.[3,10,13,18–20] Six types of labelling were distinguished: synaptic labelling (diffuse staining), granular deposits (small scattered immunolabelled spots less than 5 μm wide), focal deposits (large, 5–50 μm wide, non-amyloid rounded positive blots), vacuolar deposits (surrounding vacuoles of spongiform change), morula-type deposits (focal, non-amyloid deposits surrounded by vacuoles of spongiform change) and kuru-type amyloid plaques, 10–50 μm wide (plaques were also identified on Congo red and periodic acid Schiff-stained sections).[14,21] We did not consider other types of labelling as significant, such as that of isolated neurons or astrocytes. PrP immunostaining was considered as positive when at least one typical variety of labelling was observed in the frontal isocortex and/or the cerebellar vermis of the patient. In the study of tonsil biopsies, only patients with five or more secondary lymphoid follicles were considered[22,23] for immunostaining. The presence of intense immunoreactivity within germinal centres was considered as a positive result.

Evaluation of the 12F10 Automated Fast Procedure in a Series of 300 Brains and 47 Tonsil Biopsies from Patients with a Suspected Diagnosis of Human Prion Diseases

Between January 2001 and January 2006, 300 brains and 47 tonsil biopsies of patients with a suspected diagnosis of transmissible subacute encephalopathies were received at the R Escourolle Neuropathology Laboratory. PrP immunohistochemistry using the 12F10 automated fast procedure was performed on samples from frontal isocortex and cerebellar vermis, and tonsil biopsies. The slides were checked by two investigators (JJH and SH), who were unaware of the diagnosis. Interobserver agreement for the diagnosis of transmissible subacute encephalopathies was higher than 0.95. A consensus was reached by joint examination of the remaining samples. The gold standard was the neuropathological examination of various brain areas using standard staining procedures coupled with the western blot demonstration of PrP^{sc}.

Table 4 Antibodies screening with automation

Antibodies	*Number of positive patients/total*		
	All CJD forms	*AD*	*NND*
3F4 (m.p.)	20/21 (95.2%)	0/3	0/2
3F4	15/21 (71.4%)	0/3	0/2
12F10	20/21 (95.2%)	0/3	0/2
3B5	18/21 (85.7%)	3/3	1/2*
SAF34	18/21 (85.7%)	3/3	1/2*
BAR233	10/21 (47.6%)	0/3	0/2
4F2	19/21 (90.5%)	3/3	1/2*
SAF54	19/21 (90.5%)	3/3	1/2*

AD, Alzheimer disease; CJD, Creutzfeldt–Jakob disease; NND, patients who died from non-neurological diseases; m.p., manual procedures (reference antibody).
*Controls showing nonspecific background in the neuropile.

Results

Antibodies Screening

Step 1
When the 50 antibodies were screened using the 3F4 reference procedure that includes four pretreatments, we found a strong immunoreactivity with three antibodies that had been produced using human recombinant PrP (4F2, 3B5, 12F10), two produced using denatured scrapie-associated fibrils from 263K-infected Syrian hamsters (SAF34, SAF54) and one produced using ovine recombinant PrP (BAR233) (Table 3). Working concentrations varied between 0.8 and 2 μg/ml.

Step 2
When only one pretreatment (hydrated autoclaving) was used, a strong immunoreactivity was preserved

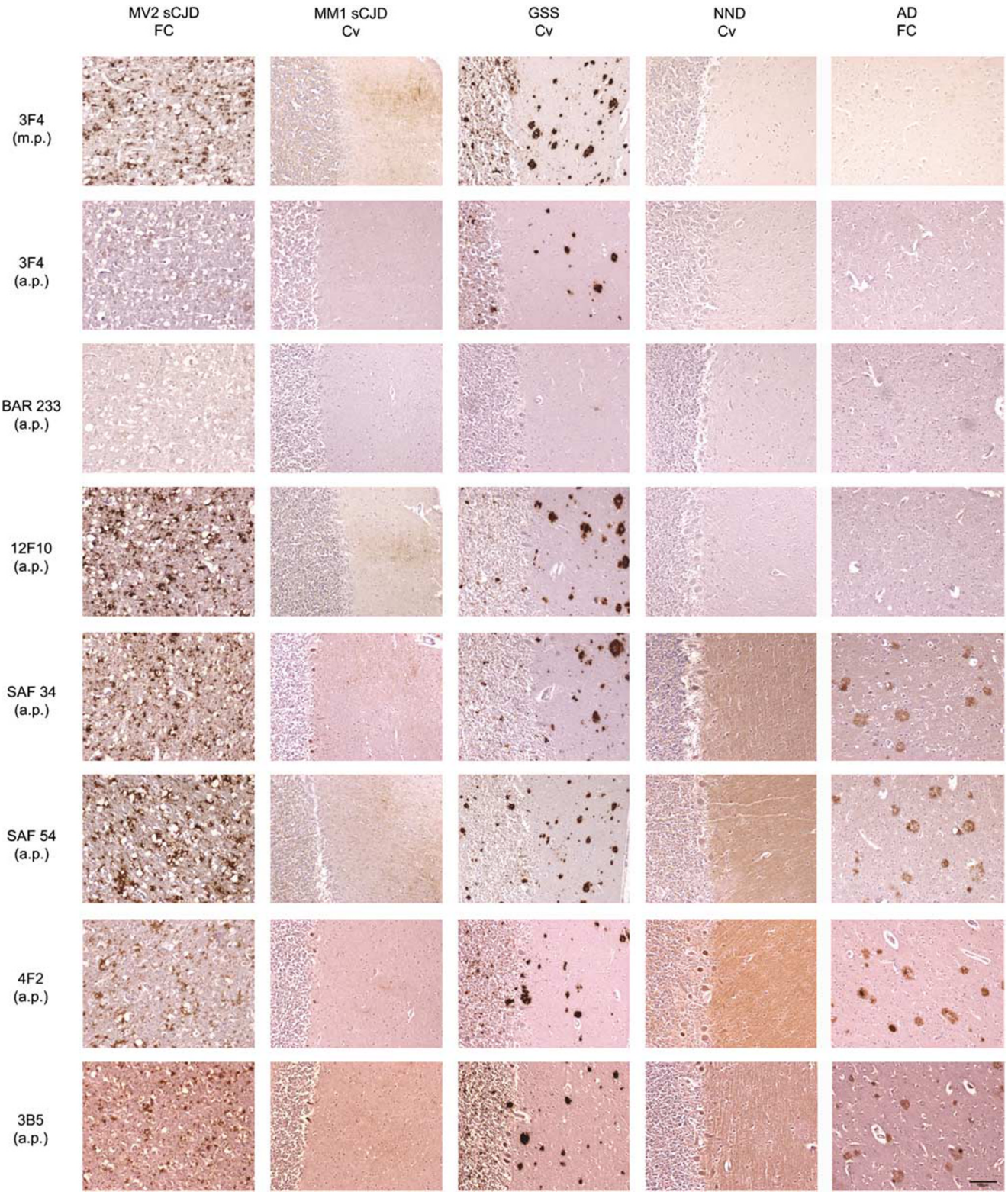

Figure 2 Immunostaining obtained with reference antibody 3F4 using manual procedure compared to 3F4, BAR233, 12F10, SAF34, SAf54, 4F2 and 3B5 antibodies using automated procedure. AD, Alzheimer disease patient; a.p., automated procedure; Cv, cerebellar vermis; FC, frontal isocortex; GSS, Gerstmann–Sträussler–Scheinker syndrome with the P102L mutation; MM1, methionine homozygosity at codon 129 of PRNP and a PrP^{sc} type 1; m.p., manual procedure; MV2, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 2A; NND, patient dead from a non-neurological disease; sCJD, sporadic Creutzfeldt–Jakob disease. Scale bar = 100 μm.

for the six antibodies selected in step 1 (Table 3). As expected, when the number of pretreatments was reduced, the staining obtained using 3F4 antibody dramatically decreased.

Step 3
The 12F10, 3B5, 4F2, SAF34 and SAF54 antibodies used in automated procedure provided a strong immunoreactivity, regardless of the pattern of PrP deposits. The 3F4 reference antibody did not work efficiently in the Nexes automated system (71.4% sensitivity) (Table 4, Figure 2). Only the fatal familial insomnia (FFI) patient yielded negative results. There were two false-negative patients using 4F2 and SAF54 antibodies (90.5% sensitivity) and three false-negative patients using 3B5 and SAF34 antibodies (85.7% sensitivity). BAR233 showed the lowest sensitivity (47.6%). The 3B5, SAF34, 4F2 and SAF54 antibodies showed background on the neuropile and positive staining on the senile plaques of the three Alzheimer disease (AD) patients tested (Figure 2). When used in the automated system, 12F10 was the most efficient antibody: sensitivity (95.5%) and deposit patterns were similar to those of manual procedure of the 3F4 reference antibody (Table 4, Figures 2 and 3).

Step 4
We compared the results of 3F4 manual procedure to the 12F10 automated one using 51 patients (Table 5, Figure 3). The sensitivity of the 12F10 procedure was slightly higher than that of the 3F4 (95.7 *vs* 91.3%). In this step, we observed the characteristic patterns of the various studied transmissible subacute encephalopathies forms, regardless of the immunostaining procedure (Figure 3).

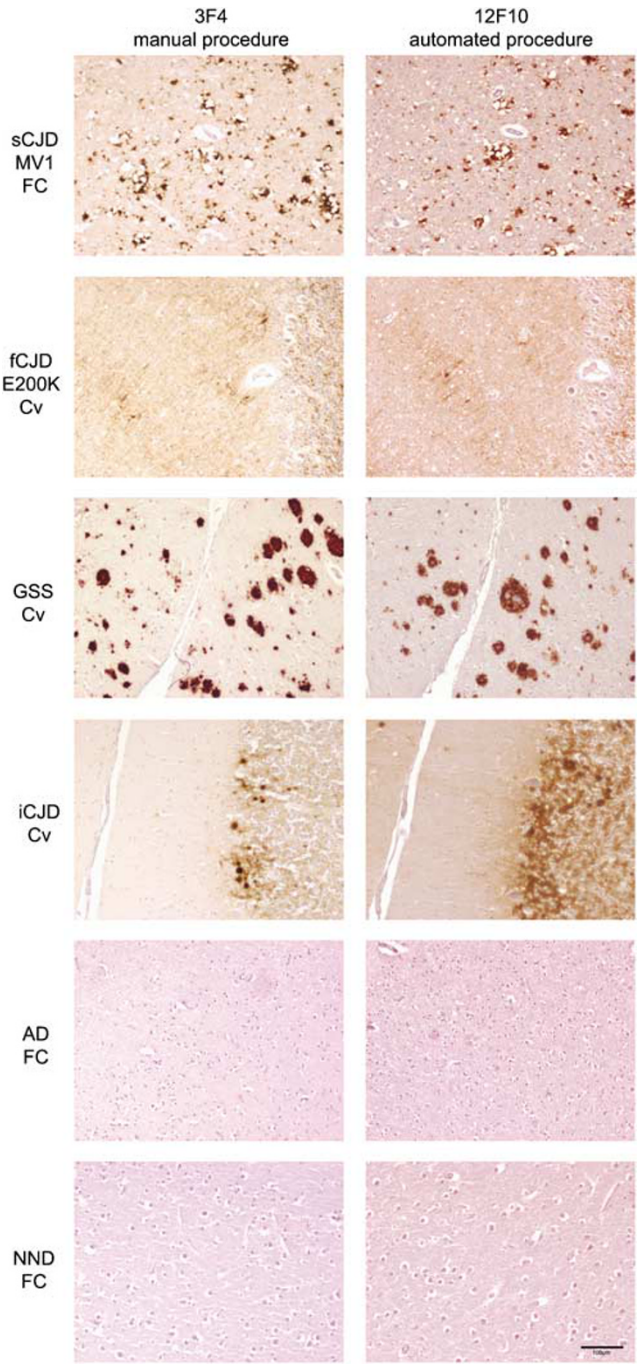

Figure 3 Immunostaining obtained with reference 3F4 antibody using a manual procedure compared to 12F10 antibody using automated procedure. AD, Alzheimer disease; Cv, cerebellar vermis; fCJD E200K, familial Creutzfeldt–Jakob disease with the E200K mutation; FC, frontal isocortex; iCJD, iatrogenic Creutzfeldt–Jakob disease after extractive growth hormone treatment; MV1, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 1; NND, patient dead from a non-neurological disease; sCJD, sporadic Creutzfeldt–Jakob disease. Scale bar = 100 μm.

Evaluation of the 12F10 Automated Fast Procedure in a Series of 300 Patients with a Suspected Diagnosis of Human Transmissible Subacute Encephalopathies

To further validate the use of 12F10 antibody in an automated procedure for the diagnosis of human transmissible subacute encephalopathies, we tested tissue sections from 300 brains of patients with suspected transmissible subacute encephalopathies. Neuropathological examination and western blot demonstration of proteinase K-resistant PrP confirmed the diagnosis of transmissible subacute encephalopathies in 169 patients out of the 300 suspected patients. Among these 169 patients, 161 were found positive in the frontal isocortex ($n = 142$), the cerebellar vermis ($n = 159$) or in both samples ($n = 140$). Only eight patients were negative in the two examined areas (95.3% sensitivity) (Table 6). The 12F10 automated procedure was efficient for the detection of PrP^{sc} deposits in all forms of studied transmissible subacute encephalopathies: sporadic Creutzfeldt–Jakob disease (sCJD) ($n = 147$), iatrogenic CJD (iCJD) after extractive growth hormone treatment ($n = 5$), variant CJD (vCJD) ($n = 3$), and familial CJD (fCJD) ($n = 14$) with various mutations ($n = 10$). Only one out of the 131 patients with non-prion diseases was immunopositive (99.2% specificity).

Evaluation of the 12F10 Automated Fast Procedure in a Series of 47 Tonsil Biopsies from Patients with a Suspected Diagnosis of vCJD

To further validate the use of 12F10 antibody in an automated procedure for the diagnosis of vCJD, we tested tissue sections from 47 tonsils of patients with suspected vCJD. Among these 47 patients, the final diagnosis was definite vCJD in 7 patients, probable vCJD in 2 and alternative diagnosis in 38. The 12F10 automated fast procedure showed positive results in all patients with definite or probable vCJD (Table 6). No positive staining was observed in the tonsils of patients with an alternative diagnosis. These results matched perfectly those of western blot assay after proteinase K digestion performed on tonsil samples from each patient.

Discussion

Identification of PrP^{sc} in the central nervous tissue by PrP immunohistochemistry is a widely used technique for the diagnosis of human transmissible subacute encephalopathies. In this study, we compared 51 monoclonal antibodies (including the gold

Table 5 Validation of 12F10 automated procedure on a series of 51 patients and comparison with 3F4 reference manual procedure

Patients	*Number of positive patients/total*	
	3F4 manual procedure	*12F10 automated procedure*
sCJD MM1	17/20	19/20
sCJD MV1	8/8	8/8
sCJD MV2	3/3	3/3
sCJD VV2	6/6	6/6
vCJD	3/3	3/3
fCJD E200K	1/1	1/1
GSS P102L	1/1	1/1
FFI D178N-129M	0/1	0/1
iCJD	3/3	3/3
AD	0/3	0/3
NND	0/2	0/2
All CJD forms	42/46	44/46
	91.30%	95.70%

AD, Alzheimer disease patient; iCJD, iatrogenic CJD after extractive growth hormone treatment; fCJD, inherited Creutzfeldt–Jakob disease with the E200K mutation; FFI, familial fatal insomnia with the D178N-129M mutation; MM1, methionine homozygosity at codon 129 of PRNP and a PrP^{sc} type 1; GSS, Gerstmann–Sträussler–Scheinker syndrome with the P102L mutation; MV1, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 1; MV2, methionine/valine heterozygosity at codon 129 and a PrP^{sc} type 2A; NND, patients dead from non-neurological diseases; VV2, valine homozygosity at the codon 129 and a PrP^{sc} type 2A; sCJD, sporadic Creutzfeldt–Jakob disease; vCJD, variant Creutzfeldt–Jakob disease.

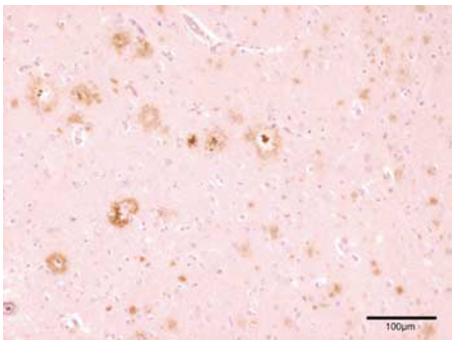

Figure 4 Immunostaining obtained with 12F10 automated procedure showing positivities within β-amyloid deposits in the frontal isocortex, from a patient with AD. Scale bar = 100 μm.

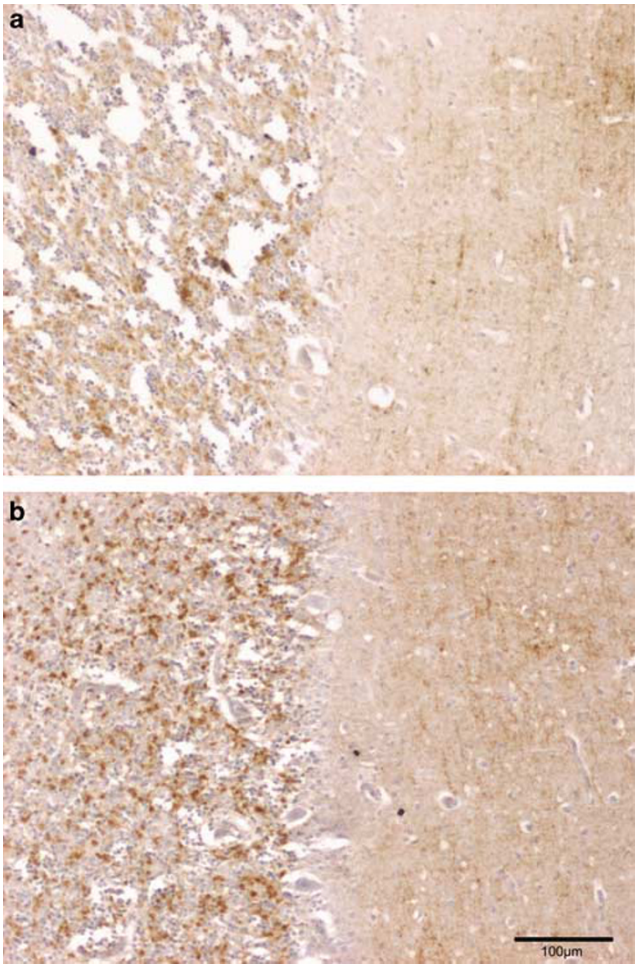

Figure 5 Tissue alterations induced by classical pretreatments used in 3F4 manual procedure (**a**) compared to only one pretreatment used in 12F10 automated procedure (**b**) on serial sections of the cerebellar vermis from a patient with sporadic Creutzfeldt–Jakob disease. Scale bar = 100 μm.

Table 6 Evaluation of the 12F10 automated procedures during a 5-year period in suspected CJD patients

	Real positive	*Real negative*	*False positive*	*False negative*	*Sensitivity in %*	*Specificity in %*
PrP immunodetection on brain	161	130	1	8	95.3	99.2
PrP immunodetection on tonsil biopsy	9	38	0	0	100	100

standard 3F4 antibody) and we developed a fast, reliable and automated procedure for PrP immunohistochemistry, allowing the diagnosis of the disease in more than 95% of the patients with a 99% specificity. Two brain areas were studied. The cerebellar vermis proved to be more often positive than the frontal isocortex.

It is worth noting that the epitopes of the antibodies that worked with limited pretreatment were located within the 59–161 region of the protein. The epitope 59–89 (4F2, 3B5 and SAF34 antibodies) and epitopes 144-152 (12F10), 157-161 (SAF54) and 141-151 (BAR233) flank the hydrophobic and potentially transmembrane domain of the protein (112–135).

In a previous study, Hainfellner *et al*[24] have described an accumulation of PrP in senile plaques in some patients with AD. We also observed a PrP immunostaining in β-amyloid deposits of AD patients using 4F2, 3B5, SAF34 and SAF54 antibodies (Figure 2). This probably corresponded to nonspecific labelling because (i) all these antibodies produced a strong nonspecific background in the neuropile, and (ii) all the studied AD patients were PrP positive using these antibodies. However, from our experience, 3F4 and 12F10 antibodies can also stain β-amyloid plaques in some AD patients (Figure 4). As previously reported, no PrP^{sc} was detected using western blot in these cases. The mechanics of co-aggregation of PrP^{c} together with β-amyloid plaque compounds remains to be established.

Few monoclonal antibodies were effective when only one epitope-retrieving procedure (hydrated autoclaving) was used. This is less time-consuming and induces less tissue alterations, as illustrated in Figure 5. It also preserves epitopes of other proteins, which is beneficial for double immunostaining. The 12F10 antibody could be used at 37°C in an automated procedure and provided the most sensitive and specific results. By comparison, the 12F10 automated method was, in our hands, more sensitive than the other procedures using the reference 3F4 monoclonal antibody, which is not suitable for

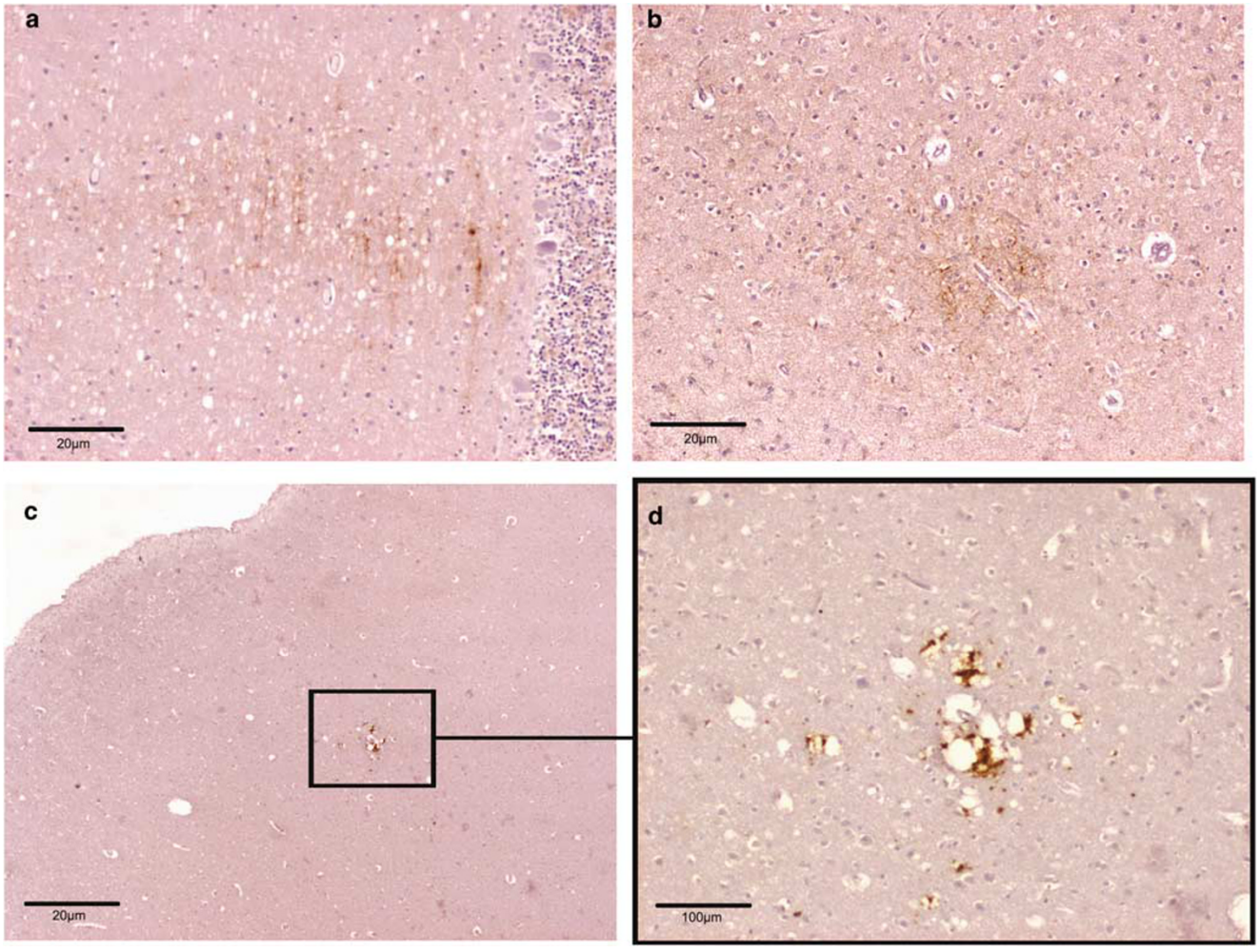

Figure 6 Immunostaining obtained with 12F10 automated procedure showing synaptic focal PrP deposits in the cerebellar vermis (**a**), frontal isocortex (**b**) and vacuolar focal deposits in frontal isocortex at different magnifications (**c**, **d**). Sections from a patient with sporadic Creutzfeldt–Jakob disease.

this automated system. Our results confirm and extend the results of Kovacs *et al*,[25] who identified 12F10 as a potentially useful antibody for immunodiagnosis, owing to a reduction in the number of pretreatments and a speeding up of the procedure using a fast immunodiagnosis automate. In addition, we demonstrated the usefulness of this procedure in a very large panel of patients affected by a high diversity of human prion disorders.

It is worth noting that PrP immunostaining interpretation is sometimes difficult because PrP deposits may be very focal within the specimen. This is the case for synaptic deposits in the cerebellar or frontal cortex of some sCJD patients with methionine homozygosity and type 1 PrP^{sc} (Figure 6). Of the 169 patients with prion diseases, 12 (7.1%) showed such focal deposits that may be difficult to identify for a pathologist who has not been trained in this technique of PrP immunohistochemistry.

This fast, automated procedure can be used for lymphoid tissue such as tonsil biopsy (nine tonsils from confirmed vCJD patients showed positive results consistently with the presence of type 2B PrP^{sc} on western blot) (Figure 7).

Among 161 patients with transmissible subacute encephalopathies, only 8 showed negative immunohistochemical results (one patient with FFI, two fCJD patients associated with the D178N-129M or the V180I mutation and five patients with sCJD). The low level of PrP^{sc} accumulation in FFI patients is a well-documented phenomenon.[26] In all the eight transmissible subacute encephalopathy patients with negative immunohistochemical results, the western blot study yielded a positive result. Different methods have been developed, such as conformation-dependent immunoassay (CDI)[27] or protein misfolding cyclic amplification (PMCA),[28,29] to increase the sensitivity of PrP^{sc} detection. How these methods could improve routine diagnosis procedure in human pathology has to be confirmed using large case–control series. In terms of diagnosis of human transmissible subacute encephalopathies, developing highly sensitive methods such as CDI and PMCA may be more adapted to prion detection in peripheral tissue where very low PrP^{sc} levels do occur (human blood). Considering our results using a fast (2 h), automated and highly sensitive procedure, more complex protocols would only slightly increase the sensitivity of detection. In addition, this procedure can be routinely applied in a general pathology laboratory.

In conclusion, after screening a large panel of anti-PrP monoclonal antibodies, we developed a fast, reliable and automated procedure for PrP^{sc} immunohistochemistry in human tissue sections. It allows a high sensitivity and specificity for the diagnosis of human prion diseases. The morphological information is preserved thanks to a simplified antigen-retrieving procedure. This procedure was effective to detect PrP^{sc} in the brain of almost all forms of human transmissible subacute encephalopathies. It can be successfully applied in lymphoid tissue.

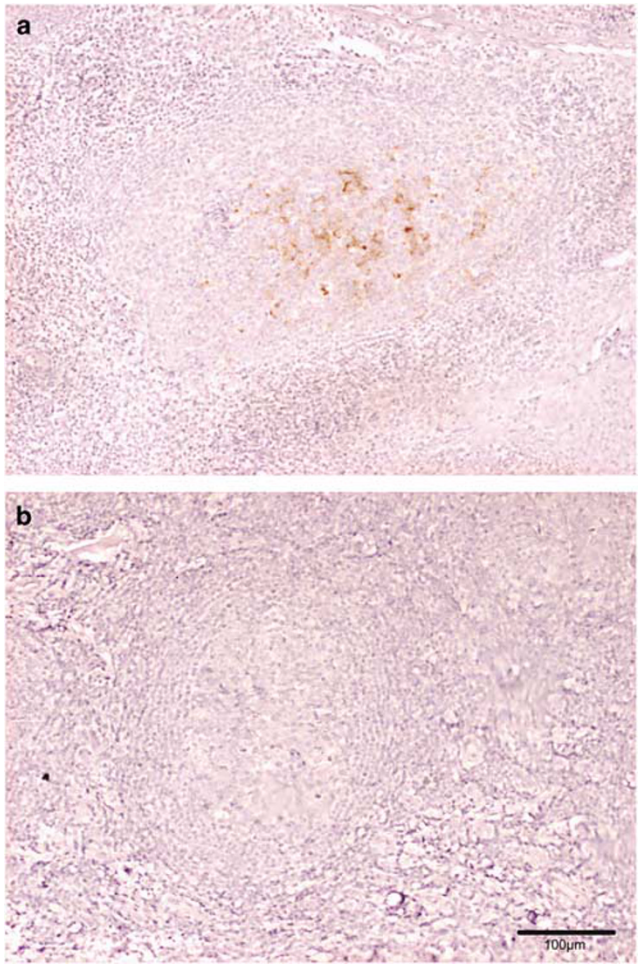

Figure 7 Immunostaining obtained with 12F10 automated procedure in tonsil biopsy sections from a patient with variant Creutzfeldt–Jakob disease (**a**) and a patient who died from a non-neurological disease (**b**).

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