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Immunohistochemical Investigations of the Prion Protein Accumulation in Human Spongiform Encephalopathies. Special Report II

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Creutzfeldt-Jakob disease (CJD) in a proportion of cases may have nonspecific clinical signs and symptoms and no characteristic neuroimaging and EEG picture. Thus, neuropathological studies are mandatory for a diagnosis. However, spongiform change, neuronal loss and astrocyte proliferation - the hallmarks of prion diseases, may also be absent or variable. In such cases, the diagnosis should be supported by the detection of prion protein (PrP) by Western blotting or immunohistochemistry (ICC). PrP may not be visualised under "regular" conditions, but it is unmasked following pretreatment procedures: incubation in formic acid or guanidine thiocyanate, microwave treatment, and hydrated or hydrolytic autoclaving, and these methods were included in standard diagnostic procedures in several different protocols. The aim of this study was to compare the effectiveness of these pretreatment methods and to introduce an optimal protocol for our laboratory. For this purpose, we used brain sections of 11 cases of CJD, 1 case of Gerstmann-Sträussler-Scheinker syndrome (GSS), 1 case of *kuru* and 3 control brains. For pretreatment we used the hydrated and hydrolytic autoclaving and incubation with formic acid. Immunostaining was performed with monoclonal 3F4 antibody against PrP. The best results were achieved with hydrolytic autoclaving. By this procedure we were able to detect the "synaptic" type of PrP accumulation in all CJD cases, as well as in GSS and kuru, while with other two methods the signal was weaker or even absent.

Introduction

Creutzfeldt-Jakob disease (CJD) is classified among transmissible spongiform encephalopathies (TSE), also known as prion diseases or transmissible cerebral amyloidoses (TCA). The term CJD covers sporadic, familial and iatrogenic forms, as well as variant CJD which have resulted from BSE infection [4, 11, 33, 59]. In addition to CJD, human TSEs consist of k*uru*, Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI).

The etiological agent causing TSEs is named "prion" - a "proteinaceous infectious particle, which is resistant to inactivation by most procedures that modify nucleic acids" (definition by SB Prusiner [51]). Prions are composed largely if not entirely of the protein (prion protein, PrP). A normal isoform of PrP is present physiologically in all mammals [10, 44]. In prion diseases, PrP accumulated as the pathological isoform (PrP^{Sc} from scrapie or PrP^{res} from proteinase resistance) of molecular weight 33 - 35kDa which is resistant to proteinase K and insoluble in detergents [51].

According to the dominant theory, PrP^{Sc} differs from its normal isoform (PrP^{C}) by its conformation: PrP^{C} is a protein with a dominant α-helical structure while PrP^{Sc} is largely β-pleated [3, 8, 16, 46, 52, 53]. This conversion of PrPc to PrP^{Sc} may be accomplished with the help of "catalytic" factors: alien or autologous PrP^{Sc}, unknown proteins (chaperons) or still unidentified virus [34, 39]. Positive correlation between PrP^{Sc} concentration and infectivity and a reduction of infectivity after PrP hydrolysis or denaturation indicates that PrP^{Sc} may be the infectious agent, but "prion" theory, although very suggestive and honored with a Nobel prize, is not yet formally proved [34, 39].

Molecular studies on TSEs demonstrated mutations in a gene encoding PrP (*PRNP*) in all familial cases (familial CJD, GSS and FFI), while no *PRNP* mutation was found in sporadic CJD, variant and iatrogenic CJD and *kuru* [see 13, 29, 30 for references]. Phenotypic manifestations of TSEs depend not only on a given mutation but also on the polymorphism at the codon 129 [10, 49, 50]: in human CJD, there is a predominance of codon 129 homozygosity (Met/Met or Val/Val), while the general population exhibits predominantly a heterozygosity Met/Val [10, 45].

CJD occurs in 4 major forms [see 29 - 31, 48 for references]:

- sporadic CJD (sCJD), most frequent (85% of cases) with the prevalence of 1 - 2 cases per million;
- familial (=hereditary) CJD, with mutations of *PRNP* gene, which is an autosomal dominant disease and accounts for 10 - 15% of cases of CJD;
- iatrogenic CJD, resulting from transmission following corneal or dura-mater grafts, human growth hormone and gonadotropins administration, and also by electrodes and neurosurgical procedures or even liver transplantation [12];
- variant (vCJD), is a result of BSE transmission from cow to man, with peculiar clinical and neuropathological presentations.

Sporadic Creutzfeldt-Jakob disease (sCJD) occurs in both sexes, with a peak at ca 65 years [see 29, 30 for references]. However, there have been CJD affected persons of both senile age [45], and also before the 20th year of life [59]. The classical triad of clinical signs in CJD consists of dementia, myoclonic jerks and a characteristic EEG record. Dementia, myoclonic jerks and alterations in EEG are observed in almost all cases but "typical" EEG curves (periodic high-voltage sharp waves and slow waves with the residual background activity) are present merely in 70% of cases. Of note, this typical record is absent in vCJD. Other clinical signs and symptoms like cerebellar, pyramidal and extrapyramidal syndromes are frequently seen.

Neuropathology

Neurodegenerative changes appear in various parts of gray matter and even in the white matter; the hallmark of CJD-infected brains is: spongiform change, neuronal loss, astrocytosis (Fig. 1) and, in 10 - 15%, amyloid plaques.

Spongiform change (Fig. 1) has an appearance of tiny vacuoles located within the neuropil, sometimes fusing in a "morule-like" fashion or even creating massive *status spongiosus* which consists of large cavities. Spongiform change is pathognomonic for the whole group of TSEs but only if they occur in deep cortical layers [40]. Occasionally, similar spongiosis may be observed in hypoxemic brains or as *post-mortem* changes, but then they occur mostly superficially. Spongiosis also occurs in Alzheimer disease (but rarely) or in frontotemporal dementias including Pick disease; in the latter condition also in the superficial location.

In some cases of CJD, particularly of the long duration, the neuronal loss and astrocytic proliferation may entirely mask the presence of spongiform change, especially when vacuoles were not that numerous [40]. Neuronal loss may occur in every brain region, and the clinical picture may depend on these locations [40]. Astrocytic proliferation is largely a non-specific phenomenon, and astrocytes in CJD are not different from these cells in the other neurodegenerative diseases [37].

Of note, these three morphological changes may not occur with the same intensity in the same areas, and severe spongiform change may not by accompanied by equally robust astrocytic proliferation. The reverse is also true, massive astrocytosis may be evident without major spongiform change, the latter is particularly true for TSEs in animals [15].

In approximately 90% of CJD cases, PrP^{Sc} accumulates in a diffuse "synaptic" form, but not plaques, and thus its detection by routine histopathological examination is not possible. In merely 10% of CJD cases PrP accumulates in the form of plaques [35, 36]. The pattern of PrP accumulation correlates with subtypes of sCJD (see further).

Amyloid plaques may be highlighted by histochemical staining as Alcian blue, PAS, Congo red and Thioflavin S but these methods are efficacious only for classic amyloid plaques and most of these methods are ineffective when PrP-amyloid plaque is not completely formed yet and exists as preamyloid which is not yet in a β-pleated form [17, 18, 35, 36].

As mentioned, in 90% of CJD cases, PrP is accumulated in a synaptic form, undetectable by light microscopy. The major method for PrP-identification is immunoblotting: Western-blotting [13], histoblotting [57, 58] and PET-blotting [55, 56] - showing PrP-distribution within the brain. Western-blotting requires unfixed brain tissues while in most TSE-cases, the only material available is a formalin-fixed paraffin-embedded brain. Histoblotting is a much more difficult procedure [58]. For this reason, immunostaining became the most widely used diagnostic tool instead of immunoblotting [2, 9, 19, 21, 22, 32].

PrP accumulating in CJD may not be detected by routine immunohistochemical procedures, probably because of its β-pleated conformation. Thus, several pretreatment methods for PrP immunostaining were described. The hydrolytic autoclaving (autoclaving in hydrochloric acid solution), introduced by Kitamoto et al. in 1992 is one of the most efficacious methods, and following its application diffuse granular ("synaptic") PrP accumulation in CNS has been described [25, 26, 28]. It was originally thought that PrP accumulates in synapses [28], but subsequent studies demonstrated the presence of PrP in dendrites rather than synaptic terminals [23, 24].

Other pretreatment techniques are: a hydrated autoclaving [31, 32, 41, 42, 43] (performed in distilled water, without HCl), incubation with formic acid [27] (also used for decontamination of fresh brain sections [6]), or with guanidine thiocyanate [2, 14, 21, 57], or combined methods [21]. Microwaving [38] and digestion with trypsin were also performed. These methods are used with different effect in several laboratories [31], and two of them - autoclaving and incubation with formic acid are most often accepted.

Using these methods, Parchi et al. [48, 49] revealed different patterns of PrP accumulation and discriminated the following subtypes of sCJD:

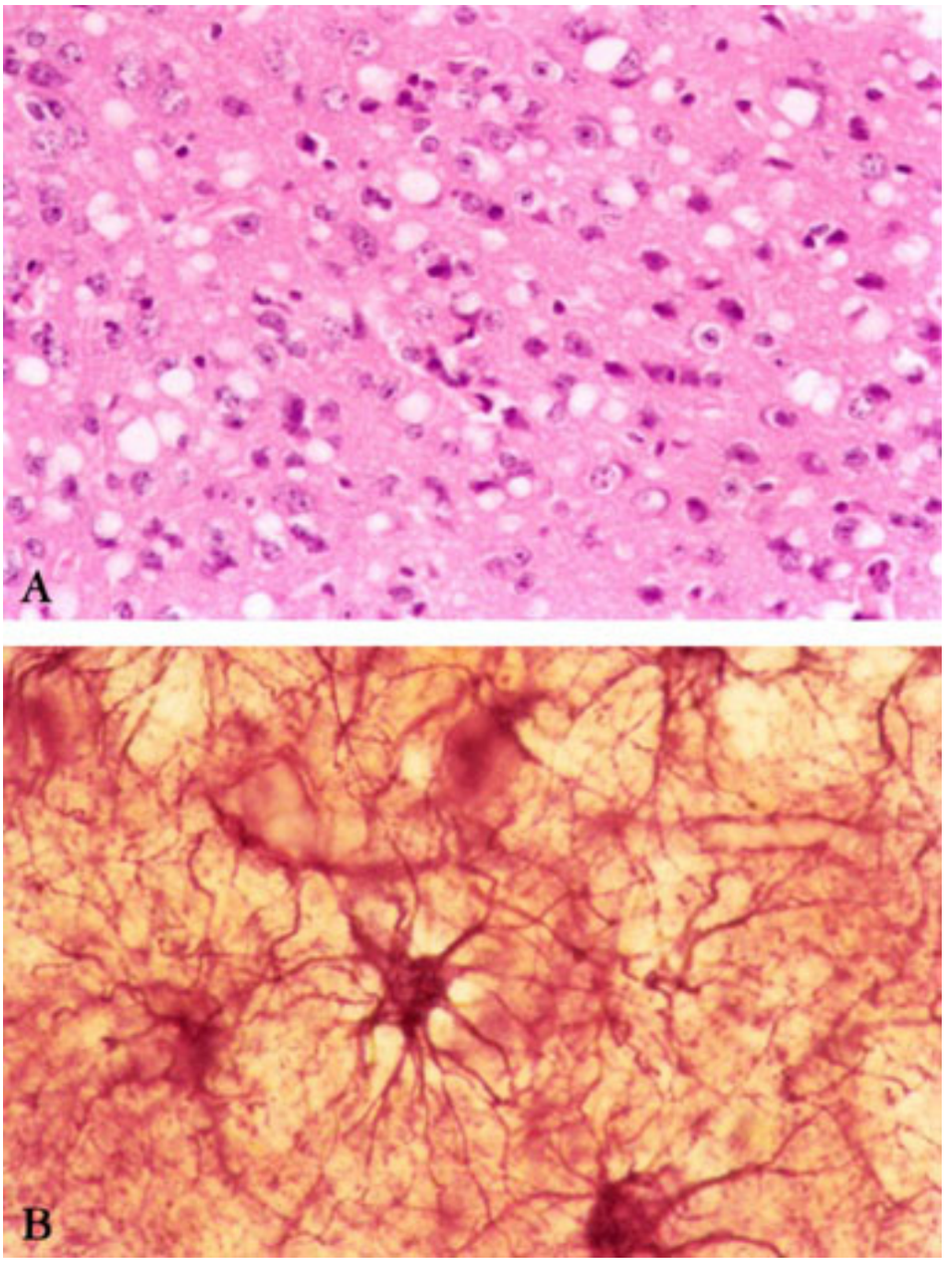

Fig. 1. A. Typical spongiform change. HE. B. Severe astrocytosis as revealed by classical Cajal gold sublimate staining.

1. Synaptic (granular), most characteristic of sporadic CJD, similar to synaptophysin immunostaining (hence, the original name);
2. Perivacuolar, where PrP accumulates between spongiform vacuoles;
3. Plaques, where PrP accumulates as *kuru*-like amyloid plaques (it is the most typical accumulation form for GSS and *kuru* [20]);
4. Plaque-like pattern, where deposits are not congophilic, usually occurring in cerebellum;
5. Laminar - in deep cortical layers.

Electrophoretic mobility of deglycosylated PrP revealed two types: PrP type 1 (21kDa) and type 2 (19kDa) and this finding coupled with the polymorphism at the codon 129 permitted further subdivision of human TSEs [49]:

1. Myoclonic type (MM1/MV1) - the most frequent sCJD subtype (70%), corresponds to the classic or the Heidenhain form. Rapidly developing disease (4 months of duration on average, maximally up to 18 months); dementia and myoclonus are dominant signs; typical EEG is always present. PrP deposits in the

TABLE 1

PrP-accumulation types (stain patterns) in tested specimens

No	Dgn.	Localisation	Pattern
1a	GSS	Cerebellum	plaque
1b	GSS	Cerebral cortex	plaque
1c	GSS	Cerebral cortex	plaque
2a	CJD	Cerebral cortex	diffuse granular synaptic; perineuronal? (Fig. 2)
2b	CJD	Cerebellum	plaque-like cerebellar; diffuse granular synaptic
2c	CJD	Frontal cortex	diffuse granular synaptic
2d	CJD	Parietal cortex	diffuse granular synaptic
3	CJD	Cerebral cortex	diffuse granular synaptic
4	CJD	Cerebral cortex	diffuse granular synaptic; perineuronal
5a	CJD	Cerebral cortex	diffuse granular synaptic
5b	CJD	Cerebellum	diffuse granular synaptic; plaque-like cerebellar
6	CJD	Cerebral cortex	perivacuolar
7	CJD	Cerebral cortex	diffuse granular synaptic; perineuronal
8	CJD	Cerebral cortex	diffuse granular synaptic
9	CJD	Cerebral cortex	diffuse granular synaptic
10	CJD	Cerebral cortex	diffuse granular synaptic
11	CJD	Cerebral cortex	perivacuolar
12	CJD	Cerebral cortex	diffuse granular synaptic
13a	*Kuru*	Cerebral cortex	diffuse granular synaptic
13b	*Kuru*	Cerebral cortex	plaque-like cortical
13c	*Kuru*	Cerebral cortex	focal granular synaptic and plaque-like
13d	*Kuru*	Medulla oblongata	focal granular synaptic
13e	*Kuru*	Cerebral cortex	focal granular synaptic
14	N-TCA	Cerebral cortex	lack
15	N-TCA	Cerebral cortex	lack
16	N-TCA	Cerebral cortex	lack, strong nonspecific background

granular ("synaptic") form in most cases, sometimes forming the perivacuolar pattern.

2. Atactic variant (VV2) with a short survival time (6.5 months). Ataxia is a dominant sign, dementia occurs later. There is no typical EEG in most cases. PrP is found as focal, plaque-like or perineuronal patterns. In the deep cortex layers, the laminar pattern is observed.
3. *Kuru* plaques variant (MV2) of the longest duration (>17 months) with dominant dementia and ataxia. No typical EEG is observed. PrP accumulates mainly in cerebellum as *kuru* plaques or plaque-like deposits.
4. Thalamic variant (MM2-T); mean duration is 15 months. Clinically manifests as fatal familial insomnia both clinically (insomnia, dysautonomia, endocrine disorders) and neuropathologically (atrophy of thalamus and olive). The sporadic form of FFI is called fatal sporadic insomnia [47, 48, 54]. Spongiform change if present is focal. PrP-accumulation is weak, and mostly in the thalamus [1].
5. Cortical variant (MM2-C) manifests as progressive dementia, which often is the only clinical feature. No typical EEG is seen. Duration is approximately 16 months. Neuropathological changes (severe spongiform change with large, confluent vacuoles) localized in the cerebral cortex; cerebellum is relatively spared. Perivacuolar PrP-accumulation pattern predominates in all cortical layers
6. Striato-cortical variant (VV1) - the rarest variant (only 1% of sCJD cases). Relatively long duration (15 months). Clinically similar to MM2-C, but with changes not only in the cortex but also in the striatum. There are no changes in the cerebellum and in brain-stem nuclei. Spongiosis without confluent vacuoles. There is only a "synaptic" PrP pattern, usually very faint.

As shown above, merely 70% of sCJD cases present the typical clinical and neuropathological picture, while remaining cases may be difficult to diagnose. Similarly, even myoclonic variants may cause diagnostic dilemmas, and PrP immunohistochemistry seems to be the most important tool to differentiate such cases.

The aim of the present study was to select the best, reproducible immunohistochemical method of pretreatment

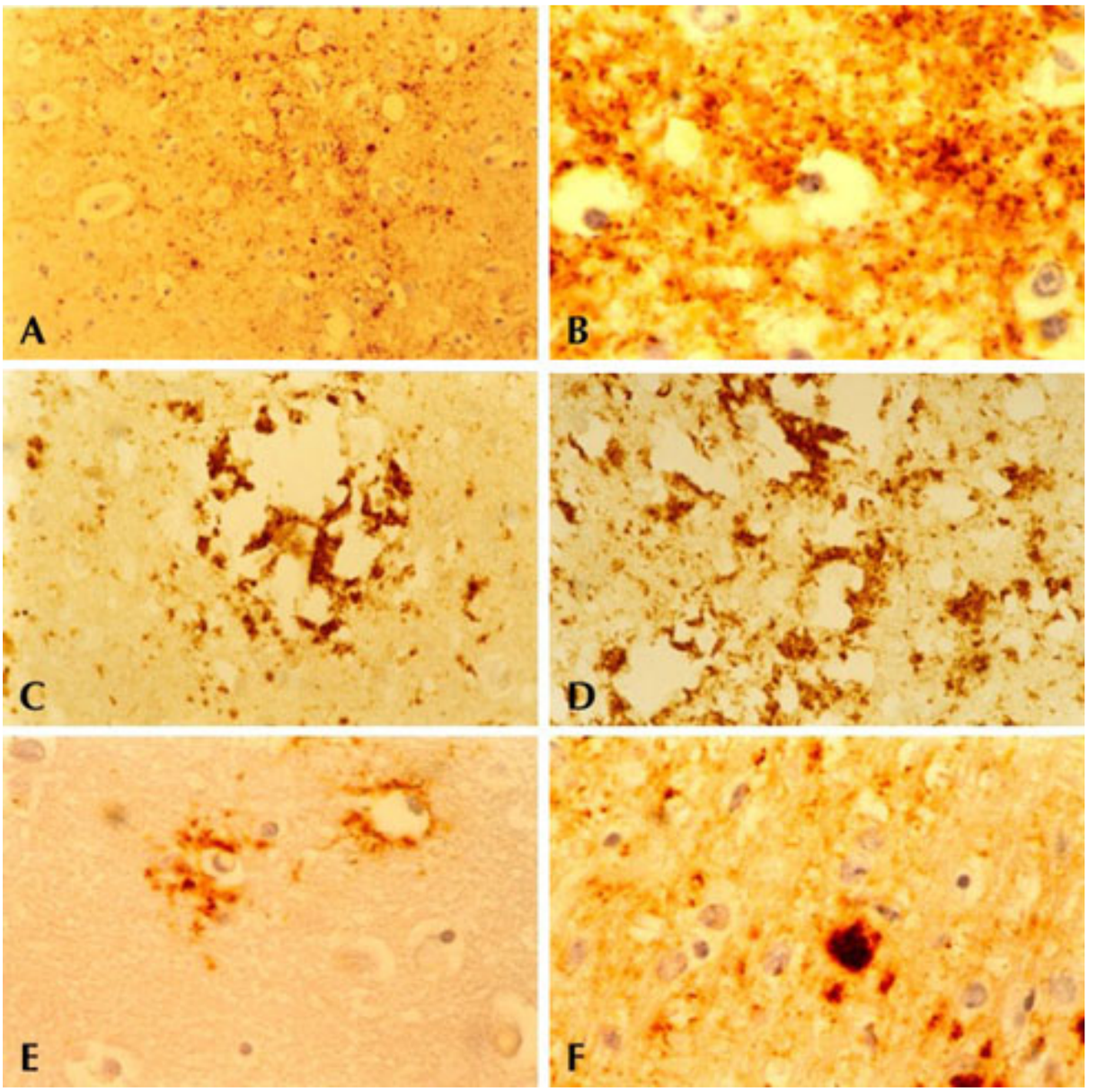

Fig. 2. Patterns of PrP staining as revealed by hydrated autoclaving and 3F4 antibodies (DAKO). A. Synaptic pattern with plaque-like accumulations. B. Robust synaptic pattern. C. Perivacuolar pattern. D. Perivacuolar pattern. E. Perineuronal pattern. F. *Kuru* plaque and smaller plaque-like deposits. Hydrated autoclaving with 3F4 Dako Abs. Magn. 1000x.

for our laboratory, which could be suggested as the standard for other Polish neuropathological departments. The other aim was to verify our cases of Creutzfeldt-Jakob disease or other transmissible spongiform encephalopathies and analyse the patterns of PrP accumulation.

Material and Methods

We used 26 formalin-fixed and paraffin-embedded brain sections of 13 clinical cases of TSEs: 11 cases of CJD, 1 case of Gerstmann-Sträussler-Scheinker syndrome (GSS) and 1 case of *kuru* [19, 35]. As the negative control we analysed 3 brain sections taken from persons who died for other TCA causes.

As a basic material for different types of pretreatment and immunostaining, we used sections of the cerebral cortex and the cerebellar cortex of a GSS brain, because of characteristic PrP amyloid plaques. As a reference pattern of diffuse type of PrP-accumulation characteristic of CJD, we used the sections of the cerebral cortex and cerebellum from persons died for CJD with a typical clinical and neuropathological presentation. These four blocks had a status of positive

control. Three cortical brain sections from 3 persons died for other diseases were used as negative control slides.

Among the methods described in the literature, the 3 most frequently used techniques were chosen: hydrated autoclaving, hydrolytic autoclaving and formic acid pretreatment. Before applying any pretreatment, sections were deparaffinized, rehydrated, washed in tap water and rinsed in distilled water. They were not incubated in formic acid before embedding in paraffin. Next, one of following pretreatments was performed:

Hydrated autoclaving

Slides were put into the metal stand and inserted into a heat-proof glass jar filled with distilled water. The jar was inserted into the steel sterilizer, and next put into "Barnstead C57830-26" autoclave. The parameters of autoclaving were: temperature - 121˚C, pressure - 140 - 145kPa (≈1.051B), exposure time - 30min.

After pretreatment, slides were left in the autoclave for 30 minutes until the pressure and the temperature had decreased.

Hydrolytic autoclaving

Instead of distilled water, hydrochloric acid was used with the concentration 1, 2, and 4mM/l. The procedure was similar, but the time of pretreatment was 10 or 30 minutes.

Formic acid pretreatment

Incubation in concentrated formic acid was performed for 15, 30, 60 and 120 minutes.

Immunostaining procedures

Following pretreatment, the slides were rinsed in distilled water. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide solution for 5 minutes. Slides were then buffered in TRIS solution for 10 minutes. As primary antibodies we used monoclonal anti-PrP sera: 6H4 or 3F4 (non-commercial obtained from Prof. H. Budka in Vienna) in dilutions 1:40, 1:80, 1:160, 1:400 and 1:800 and 3F4 (from DAKO) in dilutions 1:10, 1:20, 1:40. Slides were incubated for 1 and 2 hours at room temperature 20˚C or overnight at 4˚C.

After incubation with primary antisera the slides were washed in TRIS-buffered saline (TBS) for 10 minutes and then the incubation with kit LSAB+ (DAKO) started. The slides were then incubated in TBS for 10 minutes and immunoreactivity was visualized with diaminobenzidine (DAB). Next, the slides were washed in running tap water, rinsed in distilled water and counterstained with hematoxylin.

In the beginning of the study, positive control slides were pretreated with hydrated autoclaving (30min), hydrolytic autoclaving with three different HCl concentrations (10 and 30min) and with formic acid (15, 30, 60 and 120min), and later immunohistochemistry was performed to pick up the best serum. The strongest signal was obtained with commercial 3F4 antibody from Dako in concentration 1:50. Higher concentrations gave diminished signal. Next, all slides were pretreated as above, followed by immunohistochemistry with this antibody in dilutions 1:10, 1:20, 1:40.

Results

Efficacy of pretreatment methods

Formic acid pretreatment, for 15, 30, 60 or 120 minutes showed no positive signal in any section of CJD brain but it demonstrated a positive signal in plaques of *kuru* and GSS brains [35].

Hydrated autoclaving was helpful for the demonstration of plaques in a GSS case, but weak synaptic type of accumulation could be obtained in only a proportion of CJD brains. There were no major differences between sections incubated with antibodies of various dilutions (1:10, 1:20 and 1:40).

The intensity of staining was distinctly higher after hydrolytic autoclaving - in all CJD cases the synaptic type of immunopositivity was detected. The lowest intensity of signal was present at concentration of HCl=1mM/l. The highest intensity - at 4mM/l, but frequent section detachments or even complete destruction (fragmentation) of the slides were noted at that concentration of HCl. Pretreatment with 4mM/l HCl had also an effect on diffuse (not microgranular) non-specific background staining. The optimal results were noted at concentration of HCl=2mM/l.

Not only HCl concentration, but also time of autoclaving influenced the preservation of slides. The shortening of heating (beneficial for protection of the slides) was possible because the intensity and quality of staining were the same for 30 and for 10 minutes of autoclaving. Tests attempted without hydrogen peroxide often showed diffuse, nonspecific background staining (with various intensities). That staining was distinct, although distinguishable from the microgranular CJD-related pattern.

Positive results were obtained only following incubations with 3F4 anti-PrP antibodies with an "overnight" duration (20 - 24hrs) in a refrigerator, at stable temperature (ca. 4˚C). Attempted incubations with 3F4 Abs for 60 and 120 minutes showed no positive signals.

During autoclaving, sections often detached from the slides and after autoclaving in 4mM/l HCl this effect was particularly prominent. Some sections detached more frequently than others. By using silanized slides (Super Frost Plus), the frequency of detachment distinctly decreased. Sections incubated with formic acid also sometimes detached.

Patterns of PrP accumulation

The following major types of patterns of PrP-immunostaining were found (Table 1, Fig. 2):

1. Plaque deposits - PrP accumulates as plaques, usually large or multicentric [35];
2. Granular (or "synaptic") - diffuse, with varying density; sometimes occurs as focal granulations;
3. Perivacuolar - PrP accumulates around vacuoles;
4. Plaque-like cortical - plaque-like deposits occurring among granular (tiny) deposits, in cerebral cortex;
5. Plaque-like cerebellar - dense aggregations of granular deposits among cells of granular cell layer in cerebellum.

Besides those major types of PrP accumulation, we also observed strongly dense granular deposits and visualisation of neuronal nuclei - perineuronal stain (at the background of granular synaptic pattern).

In one of the "negative control brain" we obtained strong, diffuse labeling but without granulations. The stain was observed each time when this particular section was tested. Upon testing of other 2 "negative control slides" such a stain was never observed.

Discussion

Detection of PrP^{Sc} is mandatory for definitive diagnosis of TSE, as clinical and even neuropathological findings may not be that convincing [31]. PrP^{Sc} is not detectable in a routine histopathological examination, unless it accumulates in a form of plaques, which occurs in merely 10 - 15% of sCJD. Also even if plaques are found by routine (PAS) staining, it does not mean that it is a PrP-plaque. Thus, it is necessary to identify PrP^{Sc} with immunostaining.

Immunohistochemical studies on PrP with enhancement of immunostaining by special pretreatment were introduced in 1987 by Kitamoto et al. who used formic acid [27, 28]. Other methods of pretreatment were later described: hydrolytic autoclaving [28], hydrated autoclaving [43, 44], microwave pretreatment [41], guanidine thiocyanate pretreatment [2, 21] or combined methods [21, 31].

We report here that the most effective method of signal enhancement was hydrolytic autoclaving. Immunostaining pretreated by hydrolytic autoclaving not only produced a more intensive signal when compared with hydrated autoclaving but also shortened the autoclaving time. Most important, hydrolytic autoclaving produced a distinct "synaptic" pattern in cases, where hydrated autoclaving and formic acid pretreatment did not.

Neuropathological laboratories in the world use different methods of pretreatment: formic acid, hydrated autoclaving and hydrolytic autoclaving. Variability of effects may be demonstrated also by results showing that hydrated autoclaving is most effective and hydrolytic autoclaving is less useful method, that is in contrast to our results [20].

Hayward et al. [21] compared 8 different techniques (and their combinations) and 3 different antibodies, and found that stain intensity depends not only on the method of pretreatment, but on antibodies as well. Those dependencies were complicated - some techniques revealed more distinct stain with one antibody (and using the same method), less distinct if other antibody was used [21]. Hence, it is possible, that hydrolytic autoclaving gives the best result for 3F4 antibody from Dako.

The pattern of PrP accumulation is linked with TSE phenotype, but in various sections from the same brain, various patterns may occur, which we also observed [31, 48]. In our material, we observed all patterns described by Parchi: granular synaptic pattern, plaque-like structures, perivacuolar pattern, and even plaques in GSS and *kuru* [19, 31].

In some non-TSE cases, immunostaining reveals diffuse homogenous (without granulations) background staining; this was also observed in one of our cases [21]. Such a stain may be an artifact or indicate the PrP^{C} - normal, cellular isoform of PrP.

However, if there is no positive PrP^{Sc}-stain in an isolated section, the diagnosis of CJD cannot be absolutely excluded. In some brain regions, PrP^{Sc} may be strongly concentrated, in other regions - it may occur with very low intensity or not at all. Moreover, in some sections from CJD (even more in FFI or in FSI) patients, PrP may be undetectable at all [1, 49]. That is why brain sections should be sampled not only from just one brain region. For cases suspected of CJD, the wider panel of blocks is recommended: 1) superior and middle frontal gyrus; 2) precentral frontal gyrus; 3) insula region with basal ganglia; 4) hippocampus, temporal cortex and inferior horn of lateral cerebral ventricle; 5) pons; 6) substantia nigra; 7) cerebellum; 8) thalamus. Parietal and occipital cortex should also be sampled but in these regions PrP accumulates relatively rarely [48].

It is important to recall the problem of safety of PrP-investigations. Correctly decontaminated material by incubation in formic acid for one hour is not infectious [6, 7]. However during the tests, the basal safety conditions should be maintained. If blocks are not decontaminated, all tools should be sterilized, as recommended for autopsy instruments: in 0.5% sodium hypochlorite or 1 - 2N NaOH, or with steam autoclaving at 134˚C (brown). Autoclaving should be done in a steel sterilizer, to prevent spreading of the potentially infectious liquid into autoclave, and this sterilizer should also by decontaminated.

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