

DISCUSSION

PRNP polymorphisms are associated with variation in susceptibility to prion disease in humans [24], sheep [2, 4, 5, 13] and mice [34]. There have been many studies of relationships between *PRNP* polymorphisms and susceptibility to BSE in cattle [8, 12, 14, 22, 25, 28, 29, 31, 33]. However, few studies have found significant relationships between *PRNP* polymorphisms and occurrence of BSE [28]. The number of BSE-affected animals tested in the present study (6 cattle) was too small for reliable estimation of genetic relationships between bovine *PRNP* polymorphisms and occurrence of BSE, and we found no evidence of such relationships.

In the present study, we assayed for *PRNP* polymorphisms in 863 healthy Holstein cattle and 186 healthy JB cattle. The only polymorphism caused amino acid substitutions in the ORF was the octapeptide repeat. We observed only 2 SNPs, at G234A and C576T; these SNPs have frequently been observed in other studies [8, 10, 13, 17, 28, 31]. Only 2 of the 863 Holstein cattle were heterozygous for the C576T polymorphism, whereas 91 of the 186 JB cattle were heterozygous at C576T (Table 2). Similar differences in allele frequencies between the 2 cattle breeds were found at the 23- and 12-bp indels and the G234A SNP. These differences may be due to inherent properties of the cattle breeds or differences in the breeding systems used for Holstein and JB cattle. The allele frequencies of the bovine *PRNP* gene observed in the present study for JB cattle suggest a breeding system in which a limited number of bulls is used. Takasuga *et al.* [31] found 13 SNPs (including 2 amino acid substitutions) in indigenous Indonesian cattle, which carry more mutations than Holstein and JB cattle. It is generally thought that artificial insemination, which is widely used to breed dairy and beef cattle, decreases genetic variation and produces uniform genetic properties at the DNA level. Holstein and JB cattle raised in Japan appear to have fewer mutation sites in the *PRNP* gene than indigenous breeds such as indigenous Indonesian cattle [31].

Several cattle breeds have been shown to have octapeptide polymorphisms in the ORF of the *PRNP* gene [5, 22, 25, 33], and 3 alleles of the octapeptide repeat (5, 6 and 7 copies) have been reported. In the present study, none of the cattle had 7 copies of the octapeptide repeat, and the frequency of the 5-copy allele was very low (Tables 1 and 2). The predominant genotype of the octapeptide repeat in the present Holstein and JB cattle was homozygosity of the 6-copy allele. Variability of the octapeptide repeat has not been found to correlate with incidence of BSE in cattle [5, 14, 23], and no BSE-affected cattle have been found to be homozygous for the 5-copy allele. We found no significant differences in the genotype distribution of the SNPs or octapeptide polymorphisms between healthy and BSE-affected Holstein cattle (Table 2).

In the present study, we investigated DNA polymorphisms in 2 regions upstream from the ORF: a 23-bp indel in the upper region of exon 1, and a 12-bp indel in intron 1

(Fig. 1). The available evidence suggests that polymorphisms in these regions affect transcription of the *PRNP* gene [15, 18, 21]. In a previous study, the 23-bp insertion was found to occur more frequently in healthy cattle than BSE-affected cattle [28]. Among Holstein cattle in Japan, the 23-bp insertion has been found to have a lower allele frequency than the 23-bp deletion. We speculated that polymorphism of the 12-bp indel might affect expression levels of the *PRNP* gene, because the indel is in the promoter region of intron 1 and contains a putative Sp1-binding consensus sequence [9, 15, 18, 19]. It has been reported that a GC-rich region and Sp1-binding sequence upstream of exon 1 are both important factors in *PRNP* transcription [1, 15, 18], but the effects of this Sp1 sequence in intron 1 are unclear. Further research is needed to clarify the effects of the 12-bp indel on expression of the *PRNP* gene.

In the present study, we identified a *PRNP* gene mutant with a 288-bp deletion in the ORF (Figs. 1 and 2D), in a specimen from our DNA stock samples. A prion protein with such an internal deletion may confer resistance to prion disease infection or act as a dominant-negative mutant that inhibits prion propagation in the cell [17, 36]. Accurate determination of the distribution of this deletion could help clarify whether it has a preventive effect against prion disease. However, we did not detect this 288-bp deletion in the *PRNP* gene of any of the present 1049 cattle. Our stock DNA specimen with the 288-bp deletion in the *PRNP* gene was obtained from a calf (C928) with the calf form of sporadic bovine leucosis (SBL) [16]. SBL appears to be caused by a somatic mutation in immature pre-B cells, and it is unclear whether the malignant process of SBL is related to an internal deletion in the *PRNP* gene. If the internal deletion in the *PRNP* gene found in specimen C928 is caused by a somatic mutation that is related to the malignant transformation of SBL, it is unlikely that such a deletion would be found in healthy cattle. We examined the bovine *PRNP* gene from the other 7 specimens of SBL calves, but none of those specimens had the same deletion (data not shown).

In conclusion, we found an extremely small number of *PRNP* polymorphic sites in the 2 cattle breeds examined in the present study, and we found no association between these polymorphisms and BSE. Because the number of BSE-affected animals tested was small, further genetic investigations using many samples from BSE cattle can be useful for assessment of the risk of BSE in Japan.

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▶ 医薬品各条の改正点

生物薬品

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生物薬品の各条新規収載品目
および改正品目

生物薬品委員会の医薬品各条関係では、たん白質性/ペプチド性および高分子多糖性の新規収載品目の審議および既収載品目についての改正作業を行ってきた。

第十四改正日本薬局方(十四局)第一追補、第二追補を経て、第十五改正日本薬局方(十五局)に至るまで、新規収載品目は8品目、改正は9品目であった(表1)。これらのうち、第十四改正日本薬局方第一追補(十四局第一追補)にはリゾチーム塩酸塩(十四局第一追補では塩化リゾチームとして収載)が新規収載された。第十四局改正日本薬局方第二追補(十四局第二追補)では、オキシトシン、セラペプターゼの新規収載が行われるとともに、オキシトシン注射液、カリジノゲナーゼ、バソプレシン注射液、ヘパリンナトリウム、ヘパリ

ンナトリウム注射液、血清性性腺刺激ホルモン、注射用血清性性腺刺激ホルモン、絨毛性性腺刺激ホルモン(十四局第二追補では胎盤性性腺刺激ホルモンとして収載)、注射用絨毛性性腺刺激ホルモン(十四局第二追補では注射用胎盤性性腺刺激ホルモンとして収載)の改正が行われた。

生物薬品委員会の局方収載に
おける各条審議のポイントと
基本方針

生物薬品委員会の局方収載における各条審議は、表2にあげた基本方針に沿って行った。これらの基本方針は、十四局収載における基本方針に準じている。

① 合理性に基づく規格および試験方法の設定

生物薬品の各条における試験項目や規格値の設定においては、①品質の恒常性維持、②科学の進歩に即応した品質確保、品質管理、③後続品に対する品質基準の規範となる規格・試験法の設定、④同一原理の試験項目を重複させないなど、設定する各条ごとに合理性に基づく試験項目の設定、⑤簡便で精度の高い試験法への変更、⑥実測値に基づいた規格値の設定などの観点から審議を行うこととした。

表1 生物薬品の各条新規収載品目および改正品目

新規収載品目	改正品目
リゾチーム塩酸塩	カリジノゲナーゼ
ゴナドレリン酢酸塩	オキシトシン注射液
セラペプターゼ	血清性性腺刺激ホルモン
パルナパリンナトリウム	注射用血清性性腺刺激ホルモン
オキシトシン	絨毛性性腺刺激ホルモン
セルモロイキン	注射用絨毛性性腺刺激ホルモン
テセロイキン	バソプレシン注射液
注射用テセロイキン	ヘパリンナトリウム
	ヘパリンナトリウム注射液

表2 生物薬品委員会の各条審議の基本方針

- 1) 各条全体としての合理性に基づく規格および試験方法の設定
- 2) 範囲のある含量規格の設定
- 3) 性状の溶解性試験の見直し
- 4) 試験動物の使用削減の観点からみた合理的試験法の設定
 - ① バイオアッセイ法から理化学的試験法や免疫化学的方法への移行
 - ② ヒスタミンおよびヒスタミン様物質、異常毒性否定試験などの設定の意義および必要性の見直し
 - ③ 毒性試験の設定の意義および必要性の見直し
 - ④ 発熱性物質試験のエンドトキシン試験への切り替え
- 5) 比活性の独立示性値としての設定
- 6) 純度試験などに液体クロマトグラフィー法の積極的活用、電気泳動法で試験を実施する際の検出感度の保証
- 7) 血液凝固性物質、血液型物質などの設定の意義および必要性の見直し
- 8) 同種同効医薬品の収載にあたっての調和

② 範囲のある含量規格の設定

酵素製剤などの原薬については、単位質量当たりの活性について、上限値と下限値を設定する。このことにより、品質の恒常性の確保を目指す。

③ 性状の溶解性試験の見直し

たん白質やペプチド医薬品の特性から、有機溶媒などへの溶解性に関して、意義の乏しいものは削除することとし、合理的な性状を規定する。

④ 試験動物の使用削減の観点からの合理的試験法の設定

動物福祉やより合理的な試験法の採用を行うとの観点から、*in vivo*アッセイ法から*in vitro*アッセイ法や理化学的試験法などへの移行を図る。ヒスタミンやヒスタミン様物質試験や異常毒性否定試験などは、設定の意義や必要性を見直すこととした。発熱性物質試験からエンドトキシン試験への切り替えを進める。

⑤ 比活性の独立示性値としての設定

比活性は製品の特性を示す値であることから、純度試験として設定するのではなく、独立した示性値として設定することにした。

⑥ 純度試験などに液体クロマトグラフィー法の積極的活用、電気泳動法で試験を実施する際の検出感度の保証

液体クロマトグラフィー法などの技術進歩が著しいことから、純度試験などに積極的に活用していくこととした。その際、検出感度を設定し、保証していくことにした。

⑦ 血液凝固性物質、血液型物質などの設定の意義および必要性の見直し

血液凝固物質や血液型物質の混入に関する試験項目については、起源からみて混入の可能性のないものについては見直すことにした。

⑧ 同種同効医薬品の収載にあたっての調和

インスリンや成長ホルモンなどの同種同効医薬品の収載にあたっては、試験項目や規格値について、合理的な場合に調和を図ることとした。

新規収載候補品目の策定

上記の十五局の収載方針の策定と並行して、継続審議中の各条審議に加えて、新規収載候補品目の策定を行った。候補品目の策定

にあたっては、保険医療上重要な医薬品としてオーファンドラッグの指定を受けた医薬品や市場で広く使用されている医薬品、さらには米国薬局方(USP)や欧州薬局方(EP)に掲載されているなど国際的にも広く使用されている医薬品から、候補とすべき品目を選定した。さらに、標準品の策定が可能な医薬品を候補品目とすることにし、20品目を新規収載候補品目とした。十五局では、このうちパルナバリンナトリウム、セルモロイキン、テセロイキンが収載されることになった。

収載品目別の概説と審議の経緯の要点

① リゾチーム塩酸塩

リゾチームはムコ多糖分解作用をもつ酵素であり、溶菌活性を示す。リゾチーム塩酸塩の本質はニワトリ卵白より精製される塩基性ポリペプチドである。消炎酵素剤として用いられ、単味あるいは感冒薬に配合して用いられている。さらに、歯科領域などの小手術の止血時、点眼剤として慢性結膜炎、外用剤として皮膚科医用などにも幅広く臨床使用されている。

リゾチーム塩酸塩は医療用、一般用として広く使用され、品目数としても多数に上っている。日本薬局方外医薬品規格(局外規)に収載されていた品目であり、第十四局第一追補に収載された。

基原、性状、確認試験、純度試験、乾燥減量、強熱残分、定量法を規定した。性状についてはエーテルへの溶解性の情報は意義が乏しいことから、水およびエタノールへの溶解性を記載することとした。確認試験では、ニンヒドリンによる呈色反応および参照スペクトルとの一致を規定した。純度試験として、局外規では、溶状、重金属、ヒ素、および窒

素を規定していたが、製造工程からヒ素を規定する意義は乏しいとして、原案ではヒ素の規定を除くことが提案された。審議の結果、ヒ素を規定する必要はないとされた。一方、窒素については示性値として設定すべきとして、純度試験から独立した示性値として規定することとし、幅をもつ規格値とした。定量法としては、迅速分析法の記載に従い *Micrococcus luteus* を基質として、その溶菌活性で規定することとした。局外規では、同様に溶菌活性を指標とする定量法を採用していたが、反応停止法が規定されていなかった。そこで、定量法として反応停止を含めて規定することにより、より精度の高い試験法を採用することとした。保存条件としては、気密容器で保存可能であり、冷所などの温度規定は必要とされた。

② ギナドレリン酢酸塩

ギナドレリン酢酸塩は、ギナドトロピン(LH, FSH)分泌促進作用をもつペプチドで、視床下部性腺機能低下症、ギナドトロピン分泌不全を伴う下垂体性小人症、視床下部器質性障害、ギナドトロピン単独欠損症の治療や下垂体性LH分泌機能検査薬として用いられる。

基原、性状、確認試験、施光度、pH、構成アミノ酸、純度試験、水分、定量法を規定した。性状について、水、メタノール、酢酸、エタノールに対する溶解性を規定した。確認試験として、紫外可視吸収スペクトルと赤外吸収スペクトルを採用し、参照スペクトルとの同等性を確認することとした。また、本品は酢酸塩であることから、酢酸を含むことを確認することとした。液体クロマトグラフィー法を用いた構成アミノ酸分析法を採用し、アルギニンに対する各構成アミノ酸の比を規定した。純度試験として、溶状を紫外可

視吸光度測定法を用いた350nmにおける吸光度を限度値として規定した。また、液体クロマトグラフィー法を用いた類縁物質の限度値を規格した。定量法として、生物活性試験に替えて、液体クロマトグラフィー法を採用し、ゴナドレリン酢酸塩標準品を基準としてゴナドレリン量を求めることとした。ゴナドレリン酢酸塩は、吸湿性があり、また光感受性もあるため遮光して、冷所、密封容器に保存することとされた。

一方、ゴナドレリン酢酸塩は現在のところ、製造しているのは1社のみであり、供給できる標準品が非常にわずかしかなく、受け入れ試験として吸湿性のある本品の水分含量の測定や他の項目を十分に試験するだけの量の確保が困難との報告があった。生物薬品では、各条に収載される製品で1社のみが製造しているものが増加してくることが予想され、標準品の策定においても特別の配慮が必要と考えられた。このため、標準品の試験として必ずしも必要と考えられない試験項目については、設定しないこととした。また使用量などが必要以上に多い項目については適切な量を用いるように変更することとした。確認試験として、質量分析法を用いた分子量の規定、およびゴナドレリン酢酸塩のペプチド配列を確認できるMS/MS分析(質量分析)を行うこととした。さらに、標準品の配布制限も行うこととした。

③ セラペプターゼ

セラチア属菌から製造されたたん白質分解活性を有する酵素であり、抗炎症作用を有する。手術後や外傷時の抗炎症作用、副鼻腔炎や気管支炎の消炎、喀痰喀出不全に対する治療を目的として内服で用いる。

基原、性状、確認試験、純度試験、乾燥減量、強熱残分、定量法を規定した。SDSポリ

アクリルアミド電気泳動や免疫化学的手法を用いた確認試験の設定を要望したところ、金属プロテアーゼの特性を利用した方法が原案作成者より提案された。検討の結果、提案は適切であるとされ、定量法を準用した方法を規定することとした。原案では、重金属およびヒ素に対して独立した試験が規定されていたが、これらは純度試験として規定するべきとされた。とくに重金属に関しては実測値に基づき、より適切な規格(50ppmから20ppm)にすることとされた。定量法として、乳性カゼインの分解活性をトリクロロ酢酸可溶性画分のチロジン残基の増加を指標とする試験法により設定した。貯法としては気密容器とした。

④ パルナバリンナトリウム

ブタ腸粘膜由来のヘパリンを化学的に分解して得たヘパリンのナトリウム塩であり、血液中のアンチトロンビンⅢ(ATⅢ)を活性化することにより血液凝固阻止作用を発現する。血液透析や血液ろ過などの血液体外循環時の灌流血液の凝固防止を目的として使用されている。

基原、性状、確認試験、pH、純度試験、乾燥減量、分子量、分子量分布、硫酸エステル化の度合、総窒素、抗第Ⅱa因子活性、抗第Ⅹa因子活性・抗第Ⅱa因子活性比、定量法を規定した。基原に関して、ダルテパリンナトリウムの整合を図り、参考情報の「日本薬局方の通則等に規定する動物由来医薬品起源としての動物に求められる要件」との対応から、原料を得る動物の表記に「健康な動物」と記載することとされた。原案では試験溶媒として用いていない、エタノール(95)およびエーテルへの溶解性について記載されていたが、意義は低いとして削除することとされ、エタノール(99.5)への溶解性を追加することとし

た。確認試験として、トリジンブルーOの反応を用いた多糖類染色試験を設定するとともに、ナトリウム塩の定性反応を規定した。パルナパリンナトリウムは多くの分子量の異なるヘパリンナトリウムの集合体であることから、規格項目として分子量と分子量分布を設定し、分子量の項では平均分子量を規定することとした。分子量測定には「分子量測定用低分子量ヘパリン標準品」を用いることとした。ヘパリンナトリウムは、注射剤としても使用されることから「溶状」を設定した。硫酸エステル化は電位差滴定法を用いる試験法を採用した。原料のヘパリンナトリウムでは総窒素を規定していることから、総窒素の項を設定することとした。定量法は、標準品として低分子量ヘパリンを用い抗第Xa因子活性を指標とする試験法を採用し、1mg中の低分子ヘパリン単位を求めることとした。また、別に抗IIa因子活性の比活性を求め、抗第Xa因子活性・抗IIa因子活性の比を幅で規定することとした。貯法は密封容器とした。

⑤ オキシトシン

下垂体後葉から分泌されるペプチドホルモンで、化学合成により製造され、子宮収縮作用がある。子宮収縮の誘発、促進ならびに子宮出血の治療を目的とし、分娩誘発、微弱陣痛、弛緩出血などの治療に点滴静注あるいは、筋注により用いられる。オキシトシン注射液は既収載であったが、その原薬の新規収載を行った。

基原、性状、確認試験、構成アミノ酸、純度試験、水分、定量法を規定した。性状について、規格・試験法で使用する溶媒について調査し、水、エタノール(99.5)、および塩酸試液への溶解性を規定した。確認試験として、吸収スペクトル法を採用し、参照スペクトルとの同等性を確認することとした。構成アミ

ノ酸を設定したが、血圧上昇成分であるバソプレシン様ペプチドが存在しないことを担保するために、オキシトシンに含まれないほかのアミノ酸の限度値を規定した。純度試験では、酢酸を幅規格で設定するとともに、類縁物質について規格値を設定した。定量法は、オキシトシン注射液で動物を用いた生物活性試験が規定されていたが、高速液クロマトグラフィー(HPLC)法に変更することとした。このために、オキシトシンの強制分解溶液を用いて、生物活性試験とHPLC法を比較し、両試験法に良好な相関関係が認められたことから、HPLC法の採用が妥当とされた。貯法温度は2～8℃とし、吸湿性をもつことから気密容器とした。

⑥ セルモロイキン

セルモロイキンの本質は組換えDNA技術によって作られるインターロイキン(IL)-2であり、進行腎細胞がん、悪性黒色腫、血管肉腫に点滴静注ないしは皮下投与される。

基原、性状、確認試験、pH、純度試験、酢酸アンモニウム、エンドトキシン、無菌、定量法が設定された。確認試験では、フォリン試薬を用いたたん白質としての反応性の確認、アミノ酸分析による構成アミノ酸、SDSポリアクリルアミド電気泳動を用いた分子量、ペプチドマップ試験、IL-2依存性マウスナチュラルキラー細胞の増殖活性の抗IL抗体による阻害を指標とする試験を設定した。アミノ酸分析の試験法は、第十四局第二追補参考情報アミノ酸分析法に準じた設定を行った。ペプチドマップ試験法の規格として、ピーク位置の同等性のみならず量的な同等性を確認するために溶出ピークの高さの同等性についても確認することとした。純度試験として、酵素免疫手法による大腸菌由来たん白質、SDSポリアクリルアミド電気泳動を用いた重

合体，液体クロマトグラフィー法を用いた類縁物質のそれぞれの試験を設定した。重合体以外にSDSポリアクリルアミド電気泳動で分離可能な不純物としては，大腸菌たん白質やメチオニル化体などのセルモロイキン類縁物質が考えられるが，これらは製造工程でSDSポリアクリルアミド電気泳動の検出限度以下となるとのデータが示された。したがって，重合体の試験法としてSDSポリアクリルアミド電気泳動を採用した。緩衝液成分として，酢酸アンモニウムを用いていることから，幅をもった規格として酢酸アンモニウム量を規定した。原案では発熱性物質が規定されていたが，エンドトキシンへ変更することとした。定量法として，IL-2依存性マウスナチュラルキラー細胞のセルモロイキン依存的な増殖活性を指標として，IL-2標準品を用いて定量する方法を採用した。また，別にたん白質量を測定し，比活性を求めることとした。貯法温度は， -20°C 以下とし，滅菌した気密容器を用いることとした。

⑦ テセロイキン

テセロイキンの本質は組換えDNA技術によって作られるメチオニル化IL-2であり，進行腎細胞がん，悪性黒色腫，血管肉腫などに点滴静注ないしは皮下投与される。

基原，性状，確認試験，pH，純度試験，エンドトキシン，比活性，定量法が設定された。確認試験として，IL-2中和抗体を用いた生物活性抑制の確認試験，構成アミノ酸分析法，SDSポリアクリルアミド電気泳動による分子量，等電点電気泳動法を採用した。中和抗体を用いた生物活性抑制試験では，定量法を準用し，生物活性の中和率を規格として採用した。分子量および等電点の規格は，標準品を用いるのではなく標準物質から算出した分子量や等電点の値を規格として設定するこ

ととした。純度試験として，大腸菌由来たん白質，テトラサイクリン塩酸塩，デスメチオニル体，二量体，その他の異種たん白質，酢酸を設定した。大腸菌由来たん白質は，当初イムノラジオメトリックアッセイ法を採用していたがイムノエンザイモメトリック法に変更した。

テセロイキンは，メチオニル化体であるために，メチオニンがはずれたデスメチオニル化体の量を液体クロマトグラフィー法を用いて求めることにした。二量体およびその他の異種たん白質に関して，それぞれ液体クロマトグラフィー法を用いる試験法を採用した。定量法として，IL-2依存性マウスナチュラルキラー細胞を用いた増殖能促進作用を指標とする生物活性試験を採用し，IL-2標準品を用いて力価を求めることとした。また，別にたん白質定量試験より，テセロイキンのたん白質量を求め，比活性を規格として採用した。貯法温度として -10°C 以下とし，密封容器に保存することとした。

⑧ 注射用テセロイキン

テセロイキン注射液として，基原，製法，性状，確認試験，pH，純度試験，乾燥減量，エンドトキシン，製剤均一性，不溶性異物，不溶性微粒子，無菌，定量法を規定した。確認試験として，テセロイキンの確認試験を準用し，IL-2中和抗体を用いた生物活性抑制の確認試験，およびSDSポリアクリルアミド電気泳動による分子量の試験を規定した。定量法としては，同様に，テセロイキン定量法のIL-2依存性マウスナチュラルキラー細胞を用いた増殖能促進作用を指標とする生物活性試験を準用することとした。貯法温度は，凍結を避けた 10°C 以下とし，凍結乾燥品であることから，密封容器に保存することとした。

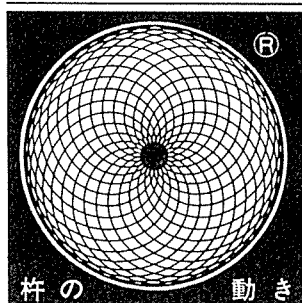
生物薬品各条審議のポイントと 生物薬品改正の基本方針と その対応

生物薬品改正の基本方針については最初に述べたが、この基本方針に沿った各条審議での対応については以下のとおりである。

ゴナドレリン酢酸塩およびオキシトシンでは当初、生物活性試験が規定されていたが、収載にあたって物理的定量法へ変更を行った。セラペプターゼの酵素製剤の原薬について、単位質量当たりの範囲のある含量規格の

設定を行うこととした。発熱性物質を規定していたセルモロイキンでは、エンドトキシンに替えることになった。また、リゾチーム塩酸塩では、試験法の見直しを行い、より精度の高い方法を採用した。また、全般的に規格値の見直しを求め、安定性を考慮した実測値に基づいた規格値を設定した。

今回の収載品目ではセルモロイキンとテセロイキンの同種同効医薬品を収載した。これらの審議にあたっては、共通の標準品を用いるものであり、各条の構成などの調和を図った。



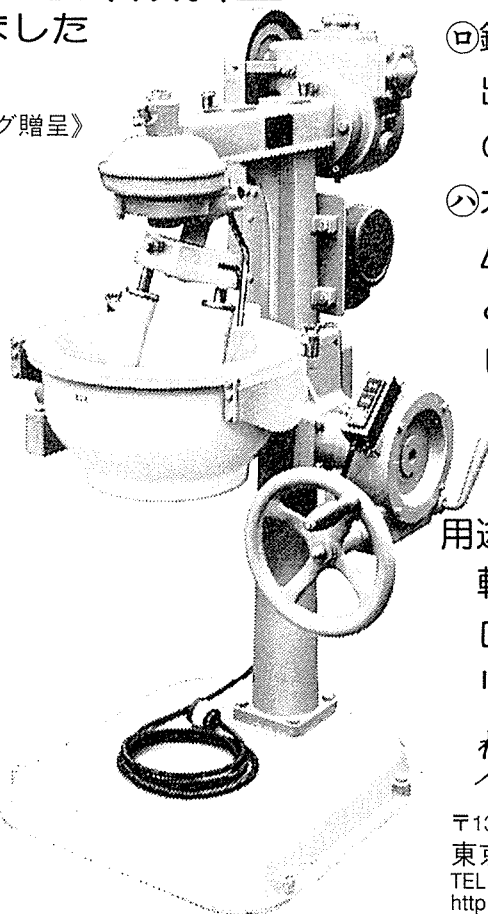
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Effective antigen-retrieval method for immunohistochemical detection of abnormal isoform of prion proteins in animals

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Abstract For immunohistochemistry of the prion diseases, several pretreatment methods to enhance the immunoreactivity of human and animal abnormal proteinase-resistant prion protein (PrP^{Sc}) on the tissue sections have been employed. The method of 121°C hydrated autoclaving pretreatment or the combination method of 121°C hydrated autoclaving with a certain chemical reagent (formic acid or proteinase K, etc) are now widely used. We found that an improved hydrated autoclaving method at 135°C, more effectively enhanced PrP^{Sc} immunoreactivity for the antibodies recognizing the linear epitope. In addition, this method was more effective for the long-term fixation samples as compared with other previous methods. However, this modified method could not retrieve PrP^{Sc} antigenic epitopes

composed of conformational structures or several discontinuous epitopes. We describe the comparative studies between our improved method and other antigen-retrieval procedures reported previously. Based on the differences of reaction among the antibodies, we also discuss the mechanisms of the hydrated autoclaving methods to retrieve PrP^{Sc} immunoreactivity.

Keywords Prion protein · Immunohistochemistry · Antigen retrieval · Autoclaving · Monoclonal antibody

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Introduction

Scrapie in sheep and goat, bovine spongiform encephalopathy (BSE), chronic wasting disease in deer and Creutzfeldt-Jakob disease (sporadic, iatrogenic, familial and variant forms) and Kuru in humans are transmissible neurodegenerative disorders belonging to a group of prion diseases. They are characterized by the accumulation of abnormal proteinase-resistant prion protein (PrP^{Sc}), which is an isoform of the cellular, proteinase-sensitive prion protein (PrP^C), as a result of post-translational modification with increases of the population of β -sheet conformation in the brain [20]. The pathology is characterized by neuronal cell loss, spongiform change, gliosis and deposition of abnormal amyloid protein.

Immunohistochemistry to demonstrate PrP^{Sc} in tissue sections is now a well-established technique for the diagnosis of prion diseases [2]. It has been reported that PrP^{Sc} immunoreactivity is enhanced by several antigen-retrieval procedures such as formic acid [4, 10], a combination of formic acid pretreatment and microwave processing [7, 14], hydrated or hydrolytic autoclaving [6, 11], guanidine thiocyanate [4, 19], and combined protocols [1, 8, 9, 15].

The recent disclosure of BSE in Japan has started an active surveillance for all slaughter cattle since October 2001. Briefly, diagnostic procedure is follows: samples

have been taken from the medulla oblongata (obex region) and examined by ELISA as the primary screening test; the ELISA-positive samples have then been confirmed by Western blot and/or immunohistochemistry. In starting an active surveillance for BSE in Japan, we applied various pretreatment methods for different antibodies to formalin-fixed and paraffin-embedded tissues to enhance PrP^{Sc} immunoreactivity. Although the pretreatment methods reported previously were found to retrieve PrP^{Sc} for antibodies used in this study, we found that an improved hydrated autoclaving method at 135°C more effectively enhanced PrP^{Sc} immunoreactivity for the antibodies recognizing the linear epitope. However, our modified method could not retrieve PrP^{Sc} antigens well for the monoclonal antibodies recognizing the conformational structures.

Here we describe the comparative studies between our improved method and other antigen-retrieval procedures reported previously, and discuss the mechanisms of the hydrated autoclaving methods to retrieve PrP^{Sc} immunoreactivity.

Materials and methods

Samples

We used the brain tissues that were cut coronally at the level including hippocampus and thalamus from two scrapie-infected and two negative control ICR mice, the medulla oblongata at the level of the obex, and the spinal cord from three scrapie-affected and two negative control sheep, and from three BSE-affected cattle in Japan and two control cattle. Two mice were inoculated intracerebrally with scrapie G1 strain, which induces amyloid plaque formations in the brain. Affected or non-affected sheep and cattle were diagnosed and confirmed by histological, immunohistochemical, and Western blot methods. These samples were fixed in 15% formalin for 48–72 h and embedded routinely in paraffin. BSE tissue blocks were treated with 98% formic acid for 1 h to reduce the infectivity of prion after formalin fixation. In

addition, we prepared the serial tissue blocks from the medulla oblongata of scrapie-affected sheep, which was immersed in 15% formalin at least for 6 months.

Immunohistochemistry

Serial tissue sections, 4 µm in thickness, were picked up on silane-coated glass slides (Muto Purechemicals Co., Japan). After deparaffinization, endogenous peroxidase was blocked by incubation in 3% H₂O₂ for 5 min. We applied six different pretreatment protocols as follows: (1) 98% formic acid for 5 min (designated as FA); (2) hydrated autoclaving at 121°C, 2 atmosphere (atm) for 20 min (with Tomy high-pressure steam sterilizer KS-323, Japan) in distilled water (121DWHA); (3) 121DWHA and 98% formic acid for 5 min (121DWHA/FA); (4) 121DWHA and proteinase K (0.4 mg/ml, Dako, USA) treatment for 1 min (121DWHA/PK); (5) hydrated autoclaving at 135°C, 3 atm for 20 min in distilled water (135DWHA); (6) 135DWHA and 98% formic acid for 5 min (135DWHA/FA). After applying each pretreatment, tissue sections were incubated with 10% goat or horse normal serum (Nichirei, Japan) for 30 min. In this study, we used the avidin-biotin complex methods (ABC kit; Vector Lab., USA) and the horseradish peroxidase-labeled polymer methods (Envision+ kit; Dako). Sections were exposed to primary antibodies for overnight at 4°C or 1 h at room temperature for ABC kit or Envision+ kit, respectively. As negative controls, the sections were exposed for each primary antibody without any pretreatments. The following steps were performed with second antibodies and others according to the each manufacture's instructions. The signals were detected using diaminobenzidine (Simple stain DAB; Nichirei, Japan). Sections were counterstained with Mayer's hematoxylin.

The characteristics of the nine primary antibodies used in this study are summarized in Table 1. For sections prepared from the tissue blocks immersed in 15% formalin for 6 months, we tested the 121DWHA and 135DWHA methods using B103 and 43C5 antibodies.

Table 1 Characteristics of the nine antibodies used in this study (*L* linear epitope, *DC* discontinuous epitope, *mAb* monoclonal antibody, *pAb* polyclonal antibody)

Antibodies	Epitope		Clonality	Dilution	Immunogen	Source
	Position	L/DC				
132	119–127	L	mAb	1:200	Mouse recPrP	Horiuchi
149	147–153	L	mAb	1:500	Mouse recPrP	Horiuchi
43C5	163–169	L	mAb	1:10000	Mouse recPrP	Horiuchi
B103	103–121	L	pAb	1:1000	Cow recPrP	Horiuchi
6H4	155–163	L	mAb	1:500	Cow recPrP	Prionics (Zürich, Switzerland)
72	89–231(143–151)	DC	mAb	1:500	Mouse recPrP	Horiuchi
44B1	155–231	DC	mAb	1:200	Mouse recPrP	Horiuchi
44B2	155–231	DC	mAb	1:200	Mouse recPrP	Horiuchi
T2	Unknown	DC	mAb	1:500	Mouse recPrP	Tagawa

Morphometry

Serial sections from BSE-affected samples were pretreated with 121DWHA, 121DWHA/FA, 121DWHA/PK, 135DWHA, and 135DWHA/FA methods, and immunostained with four antibodies (B103, 43C5, 44B1, and 6H4), respectively. Each of the pretreatment conditions were evaluated on the selected five areas (total μm^2). The Lumina Vision computer analysis system (Mitani Corp., Tokyo, Japan) was used to measure the positive area of PrP immunostaining. The brown-colored chromogen precipitate was selected, digitized images of these areas, and the digital pixels converted into the density area (μm^2) on the software. The highest density measurement was set to 100% and relative density (RD) of immunostaining by the other pretreatments in the same area was calculated.

Results

Histopathology

In the mouse, neuropil vacuolation associated with astrogliosis and microglial proliferation was observed throughout all areas of the brain. Amyloid plaque structures were also seen, which were often observed in contact with the capillary vessels.

In the obex region of scrapie-infected sheep, neuropil vacuolation and single or multiple intracytoplasmic vacuoles were particularly found in the dorsal motor nucleus of vagus nerve (DMNV), gracile nucleus, nucleus ambiguus and reticular formation [22]. The hypoglossal nucleus, olivary nucleus and nucleus of solitary tract (NST) were only mildly affected. In addition, spongiform

Table 2 Results of the immunoreactivity for the antibodies under pretreatment methods [FA 96% formic acid for 5 min, 121DWHA hydrated autoclaving at 121°C, 2 atmosphere (atm) for 20 min in distilled water, 121DWHA/FA 121DWHA and 96% formic acid for 5 min, 121DWHA/PK 121DWHA and proteinase K treatment for 1 min, 135DWHA hydrated autoclaving at 135°C, 3 atm for

20 min in distilled water, 135DWHA/FA 135DWHA and 98% formic acid for 5 min, M scrapie-affected mouse, C BSE-affected cow, S scrapie-affected sheep, P plaque type, D diffuse type, 3+ strongly positive signal, 2+ moderately positive signal, + faint positive signal, - negative]

Antibodies	Tissue source	Pretreatment					
		FA	121DWHA	121DWHA/FA	121DWHA/PK	135DWHA	135DWHA/FA
132	M/P	-	2+	2+	+	2+	2+
	M/D	-	+	2+	-	+	2+
	C	-	-	+	-	3+	3+
	S	-	-	2+	-	2+	3+
149	M/P	2+	+	2+	2+	3+	2+
	M/D	+	+	2+	+	3+	+
	C	-	-	2+	-	+	3+
	S	-	+	2+	2+	2+	3+
43C5	M/P	+	2+	2+	2+	3+	3+
	M/D	-	2+	2+	2+	2+	2+
	C	-	2+	2+	2+	2+	3+
	S	-	2+	2+	2+	3+	3+
B103	M/P	-	+	2+	2+	3+	3+
	M/D	-	+	2+	2+	3+	3+
	C	-	+	2+	2+	3+	3+
	S	-	+	2+	2+	3+	3+
6H4	M/D	-	+	2+	+	-	-
	M/P	-	+	2+	-	-	-
	C	-	+	2+	-	-	-
	S	-	+	2+	-	-	-
72	M/P	2+	+	3+	-	-	2+
	M/D	-	-	+	-	-	-
	C	+	-	2+	+	-	-
	S	-	+	2+	-	-	-
44B1	M/P	+	+	2+	-	+	2+
	M/D	-	-	2+	-	-	2+
	C	+	+	2+	-	-	+
	S	-	-	2+	-	-	+
44B2	M/P	+	+	3+	-	-	2+
	M/D	-	-	2+	-	-	+
	C	-	-	2+	-	-	-
	S	-	-	2+	-	-	-
T2	M/P	2+	+	3+	-	-	+
	M/D	-	-	2+	-	-	-
	C	-	-	2+	-	-	+
	S	-	-	2+	-	-	-

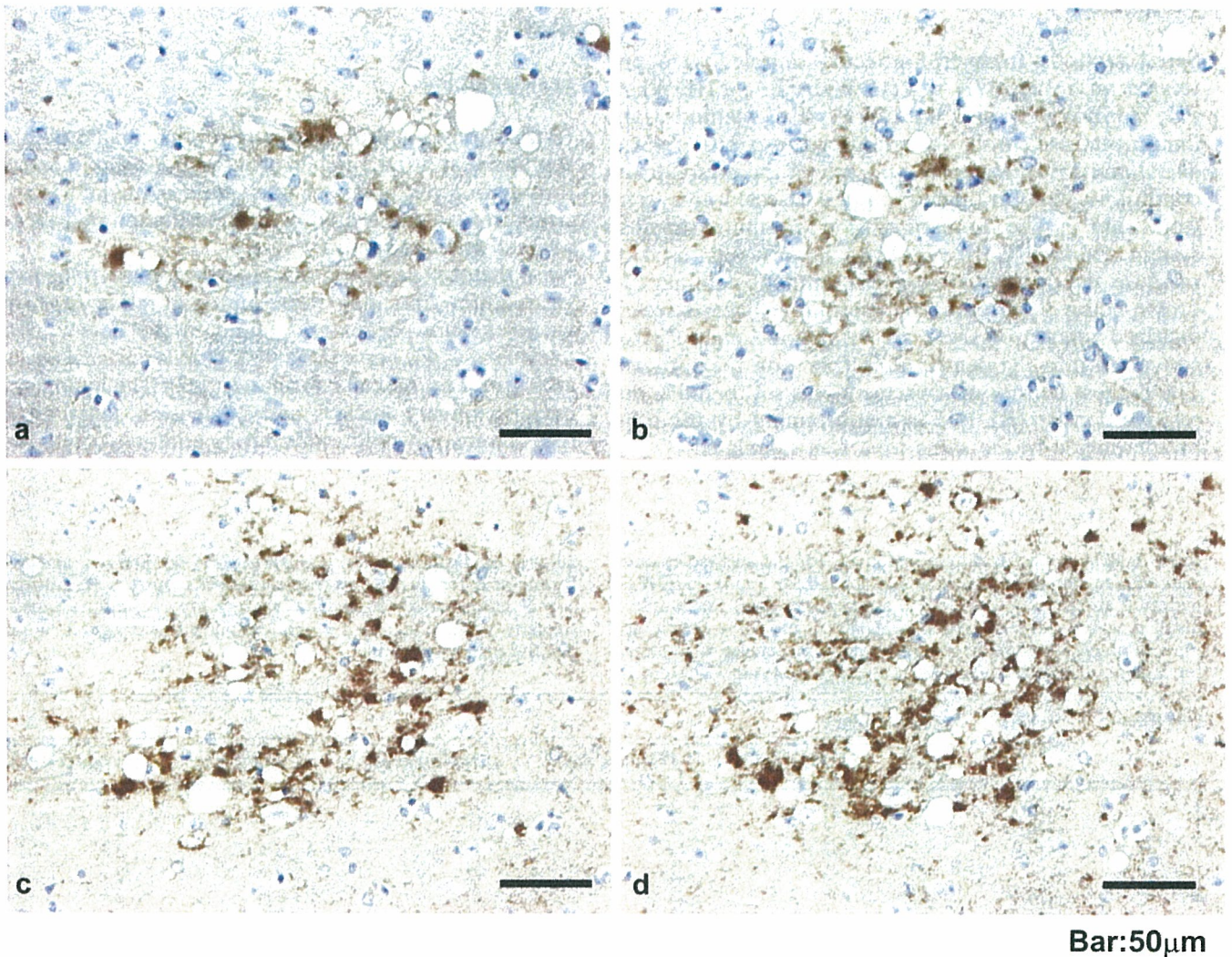


Fig. 1 Immunohistochemistry of the PrP with mAb 43C5 in the thalamus of scrapie-affected mouse; **a** 121DWHA, **b** 121DWHA/FA, **c** 135DWHA and **d** 135DWHA/FA methods. The immunodensity is considerably greater using the 135DWHA and 135DWHA/FA methods [*PrP* prion protein, 121DWHA hydrated autoclaving at 121°C, 2 atmosphere (atm) for 20 min in distilled water, 121DWHA/FA 121DWHA and 96% formic acid for 5 min, 135DWHA hydrated autoclaving at 135°C, 3 atm for 20 min in distilled water, 135DWHA/FA 135DWHA and 98% formic acid for 5 min]. Bars **a-d** 50 μ m

neuropil lesions were seen in the periphery of the dorsal column of spinal cord and the vertebral column.

In BSE cases, because of the subclinical case, extremely mild spongiform lesions were observed only in the DMNV, and periphery of the reticular formation [24].

Immunohistochemistry

Immunohistochemical examination revealed that no PrP depositions were observed in the sections from the affected animals without pretreatment and from the control animals with and without pretreatment.

PrP^{Sc} immunostaining yielded characteristic patterns in each animals affected with prion disease [21]. The following immunostaining patterns were observed: (1) fine particulate deposition; (2) coarse particulate deposition; (3) perineuronal deposition; (4) glial type deposition; (5) perivascular or perivacuolar deposition; (6) plaque or plaque-like deposition.

In scrapie-infected mice, PrP^{Sc} deposits were observed diffusely in cortex, thalamus, and hippocampus. Perivascular, perivacuolar PrP^{Sc} deposits and plaque or plaque-like structures in thalamus were also seen.

The PrP^{Sc} deposits in scrapie-affected sheep were most intense in the DMNV. The hypoglossal nucleus also showed deposits, but the staining was sparse. Glial, coarse particulate, perineuronal, perivascular and perivacuolar depositions were found in the reticular formation.

In BSE cases, the intense positive reactions of PrP^{Sc} were observed in the DMNV, NST and periphery of the reticular formation, which showed fine, perineuronal and perivacuolar patterns. Fine or coarse particulate depositions were seen in olivary nucleus. The hypoglossal nucleus also showed positive reactions, but with a low intensity of immunostaining.

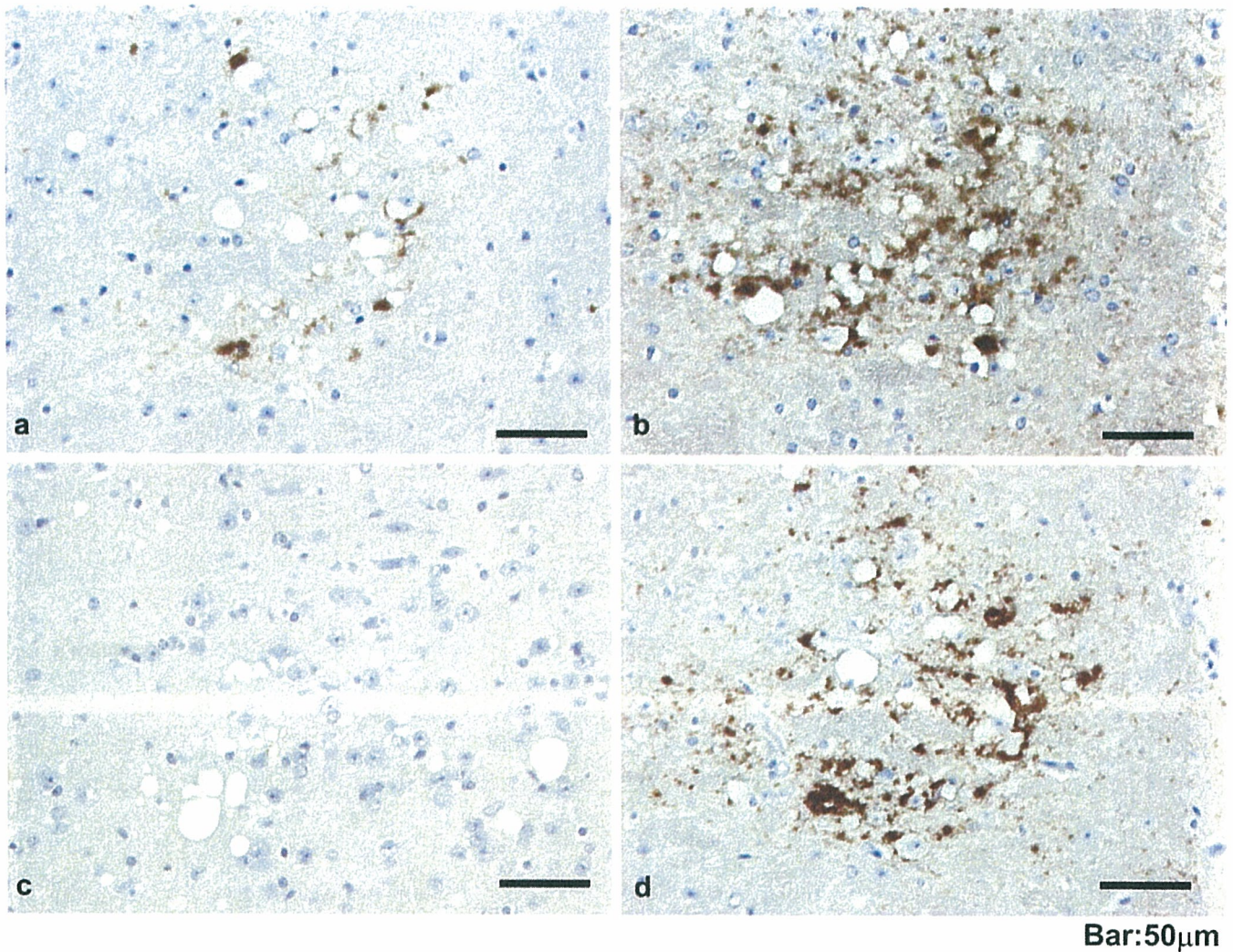


Fig. 2 Immunohistochemistry of the PrP with mAb 44B1 in the thalamus of scrapie-affected mouse; **a** 121DWHA, **b** 121DWHA/FA, **c** 135DWHA and **d** 135DWHA/FA methods. The 121DWHA/FA method is the most effective for enhancing the PrP and only faint reactivity is observed with the 121DWHA method. Although 135DWHA/FA method enhances the PrP, no reactive deposits are observed using the 135DWHA method. Bars **a–d** 50 µm

Comparison of pretreatment methods

Microscopic examinations for each pretreatment and antibody are summarized in Table 2. All antibodies used in this study reacted with PrP^{Sc} of all animals under some pretreatments.

Single FA pretreatment gave very weak or no reactions to all antibodies; however, mouse amyloid plaques were positive to some antibodies (mAbs 149, 43C5, 72, 44B1, 44B2, and T2). Among 121DWHA, and a combination of 121DWHA with FA (121DWHA/FA) or PK (121DWHA/PK) pretreatments, 121DWHA/FA was most effective with most antibodies, but 121DWHA/PK was more or equally effective compared with 121DWHA/FA using mAb 43C5 and pAb B103. Considering the sensitivity to 135DWHA and 135DWHA/FA pretreatments, the antibodies used in

this study have been divided into two types, one which showed the increase in reactivity, and the other showing a significant reduction or a loss of reactivity. mAbs 132, 149, 43C5 and pAb B103, which reacted with the linear epitope, are classified to the former group (Fig. 1a–d), while mAbs 72, 44B1, 44B2 and T2 recognizing the discontinuous epitope are referred to the latter group (Fig. 2a–d). mAb 6H4 against cow recombinant PrP stained bovine, ovine and murine PrP^{Sc} with 121DWHA/FA pretreatment, but other pretreatments showed no positive reactions for any animal PrP^{Sc} except for the murine plaque type of PrP^{Sc} with 121DWHA, 121DWHA/FA and 121DWHA/PK pretreatments.

Effects on the sections from the tissues immersed in formalin for a long period

With 121DWHA and 135DWHA pretreatment, both B103 and 43C5 antibodies gave positive reactions in almost the same areas for sections of scrapie-affected sheep that had been immersed in formalin for

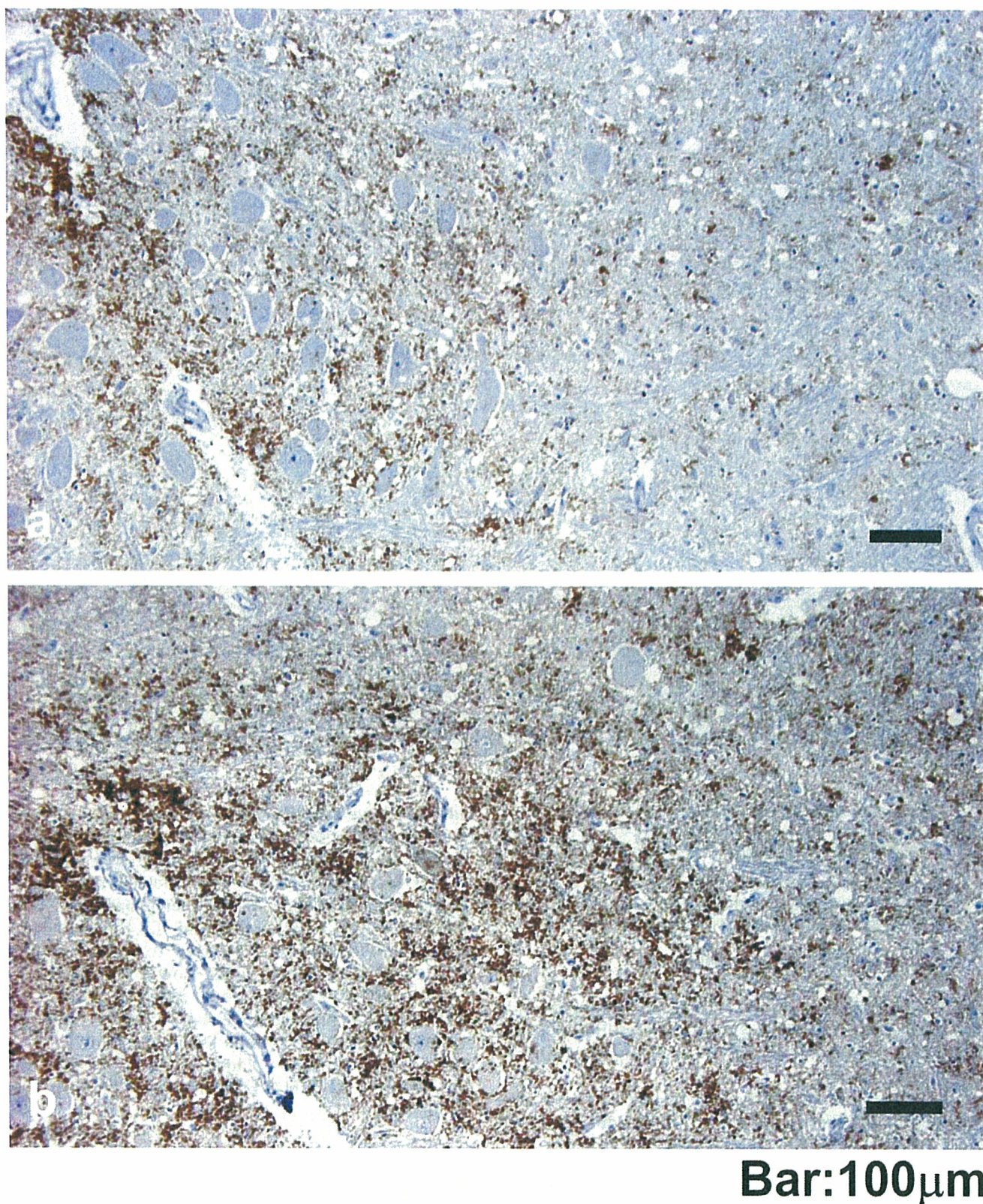


Fig. 3 Immunohistochemistry of the PrP with pAb B103 in the sections of scrapie-affected sheep from the sample immersed in formalin for 6 months; **a** 121DWHA and **b** 135DWHA methods. Bars **a**, **b** 500 µm

6 months. However, the sections treated by the 135DWHA method yielded an intense signal and widespread staining, while the detectable antigens in 121DWHA pretreatment were limited and showed low intensity (Fig. 3a, b).

Morphometry

The comparison of RD in each pretreatment method on BSE sections is shown in Fig. 4. This digital morphometry was in good agreement with the observations throughout. Although 135DWH or 135DWH/FA methods increased the density with mAb 43C5 and pAb B103, the effect was stronger with the former (Fig. 4a, b). Using mAbs 44B1 and 6H4, the 121DWH/FA protocol was appropriate for antigen retrieval and the

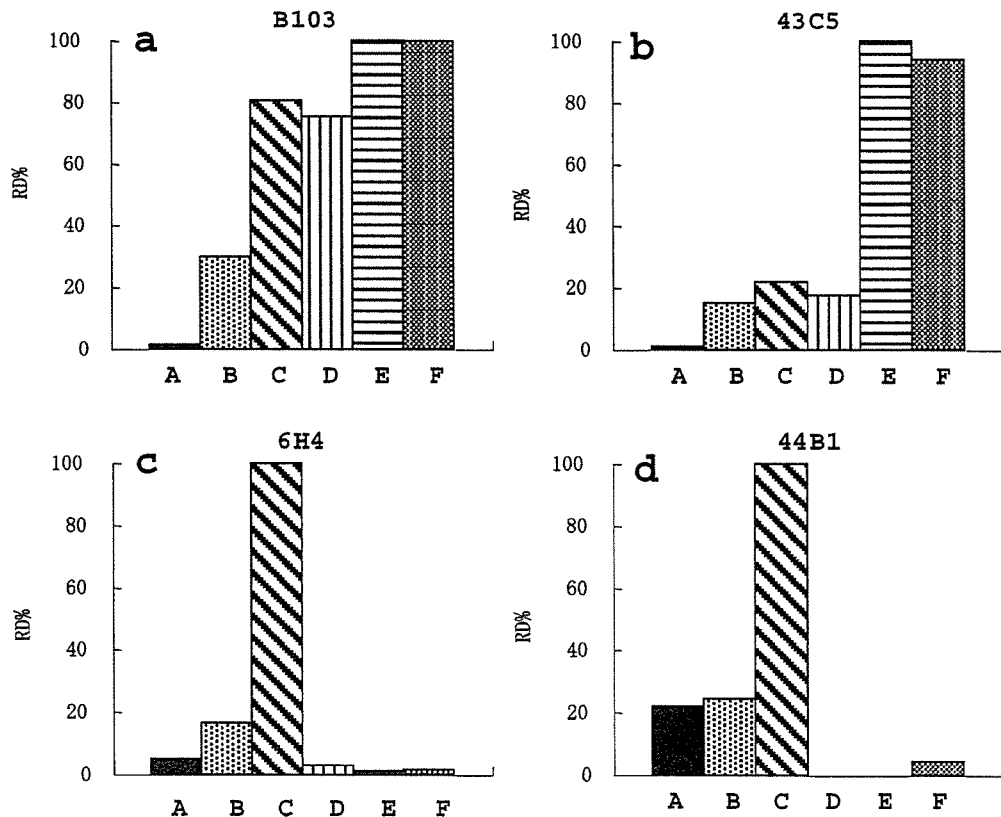
135DWH or 135DWH/FA method were not effective (Fig. 4c, d).

Discussion

In immunohistochemistry for the prion diseases, several pretreatment methods to enhance the immunoreactivity of human and animal PrP^{Sc} on the tissue sections have been reported. The method of 121°C hydrated autoclaving pretreatment or the combination method of 121°C hydrated autoclaving with a certain chemical reagent (formic acid or proteinase K, etc) are now widely used [5, 13]. The precise mechanisms by which pretreatments enhance the PrP^{Sc} immunoreactivity are still unknown. However, the effect of these chemical treatments is considered to make amyloid fibril proteins denature, breaking down the structure of amyloid fibrils, and exposing the buried epitopes [4]. In formic acid pretreatment, microwave irradiation is thought to enable for formic acid penetration in tissue, and to expose the epitope and react with the antigen more efficiently [7]. In addition, it is speculated that hydrolytic autoclaving contributes to alter the primary structures of PrP in situ [11].

We describe a new hydrated autoclaving method, termed the 135DWH method, to enhance the immunoreactivity of the PrP^{Sc}, and have compared this with the previously reported methods. Generally, the 135DWH or 135DWH/FA methods for the antibodies reacting with linear epitope showed a higher

Fig. 4 Comparison of relative density (RD%) using each pretreatment method on BSE sections for different antibodies; **a** pAb B103, **b** mAb 43C5, **c** mAb 6H4, and **d** mAb 44B1. mAb 44B1 recognizes discontinuous epitope, and others recognize linear epitope. The effect of FA, 121DWH, 121DWH/FA, 121DWH/PK, 135DWH and 135DWH/FA is displayed as bar A, B, C, D, E and F, respectively. **a** FA displays 1 RD%. 121DWH (29 RD%) does not lead to a significant increase. 121DWH/FA and 121DWH/PK show a density of 81 and 75 RD%, respectively. Immunodensity is further enhanced by 135DWH (100 RD%) and 135DWH/FA (100 RD%). **b** FA displays 1 RD%. 121DWH, 121DWH/FA and 121DWH/PK show 15, 22 and 18 RD%, respectively. 135DWH and 135DWH/FA result in 100 and 94 RD%, showing a significant increase. **c** FA (5 RD%), 121DWH (17 RD%), 121DWH/PK (3 RD%), 135DWH (1 RD%) and 135DWH/FA (2 RD%) do not lead to a significant increase in immunodensity. 121DWH/FA (100 RD%) shows the highest increase. **d** FA shows 22 RD% and 121DWH 25 RD%. Immunodensity is not enhanced by 121DWH/PK and 135DWH. 135DWH/FA displays 4 RD%. The best result was obtained by 121DWH/FA (100 RD%) (FA 96% formic acid for 5 min, 121DWH/PK 121DWH and proteinase K treatment for 1 min)



sensitivity than 121DWHA, 121DWHA/FA, or 121DWHA/PK methods, except for antibody 6H4. Although prolonged exposure of brain material to aldehyde fixatives usually dramatically decreases the antigenicity of PrP^{Sc} [17], this newly enhancing method was more effective for the long-term fixation samples compared with other methods. On the other hand, our simple modification could not enhance immunoreactivity for the prion antigen for antibodies recognizing discontinuous or conformational epitopes.

For immunohistochemical antigen-retrieval techniques, hypotheses such as breaking cross-linking [16], protein denaturation or modification-re-modification [3] have been proposed, and were thought to have an advantage on the basis of observation or support from several studies [18, 22, 23]. In particular, the later theory is based on heat- or chemical-induced modification of the three-dimensional structure of "formalinized" protein, restoring the condition of a formalin-modified protein structure back towards its original structure on the paraffin-embedding tissue sections. Because immunohistochemistry without pretreatment did not give any positive reactions using the pAb B103 and 44B1 in the frozen sections (data not shown), there are some differences between this theory and our model. However, it seemed probable that epitopes hidden by the aggregation of PrPs are exposed on the surface, or that conformational binding sites formed by the other protein molecules are disrupted due to conformational changes induced by the hydrating autoclave methods on the formalin-fixed paraffin sections, assuming that the principle of the antigen-retrieval methods is to lead to a re-naturation or partial restoration of the protein structure with re-establishment of the three-dimensional to something approaching its native condition [22, 23].

Antibody, especially that reacting on the discontinuous epitope, recognizes specific epitopes localized in a spatial configuration within the protein molecule. mAb 15B3 recognizes the discontinuous epitope in the pathological PrP isoform, and a single continuous 15B3 binding site was speculated to be formed either by aggregation of two or several PrP molecules, or by structural rearrangement of a single PrP molecule, or by a combination thereof [12]. The exact mechanisms causing the differences between 135DWHA and 121DWHA methods in the antibody's recognition of the conformational epitope are still unknown. However, these can be surmised as follows: some aggregate proteins or molecules may be loosely arranged and antigenic determinants come to lie on the surface during formic acid or 121°C, 2 atm autoclaving pretreatment; furthermore, elevation of the temperature and atmosphere may cause further changes of certain stereoscopic structures or components of PrP molecules, causing a loss of its conformational epitope. Additional formic acid treatment also causes a slight change, helping in the demasking of the conformational epitope.

Further studies on prion antigen-retrieval techniques, including establishing an exact correlation of these mechanisms and the antibody epitope, may shed new light in the pathology of the prion diseases.

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Conformational change in full-length mouse prion: A site-directed spin-labeling study

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Abstract

The structure of the mouse prion (moPrP) was studied using site-directed spin-labeling electron spin resonance (SDSL-ESR). Since a previous NMR study by Hornemann et al., [Hornemann, Korth, Oesch, Rieka, Widera, Wüthrich, Glockshuber, Recombinant full-length murine prion protein, mPrP (23–231): purification and spectroscopic characterization, *FEBS Lett.* 413 (1997) 277–281] has indicated that N96, D143, and T189 in moPrP are localized in a Cu²⁺ binding region, Helix1 and Helix2, respectively, three recombinant moPrP mutations (N96C, D143C, and T189C) were expressed in an *Escherichia coli* system, and then refolded by dialysis under low pH and purified by reverse-phase HPLC. By using the preparation, we succeeded in preserving a target cysteine residue without alteration of the α -helix structure of moPrP and were able to apply SDSL-ESR with a methane thiosulfonate spin label to the full-length prion protein. The rotational correlation times (τ) of 1.1, 3.3, and 4.8 ns were evaluated from the X-band ESR spectra at pH 7.4 and 20 °C for N96R1, D143R1, and T189R1, respectively. τ reflects the fact that the Cu²⁺ binding region is more flexible than Helix1 or Helix2. ESR spectra recorded at various temperatures revealed two phases together with a transition point at around 20 °C in D143R1 and T189R1, but not in N96R1. With the variation of pH from 4.0 to 7.8, ESR spectra of T189R1 at 20 °C showed a gradual increase of τ from 2.9 to 4.8 ns. On the other hand, the pH-dependent conformational changes in N96R1 and D143R1 were negligible. These results indicated that T189 located in Helix2 possessed a structure sensitive to physiological pH changes; simultaneously, N96 in the Cu²⁺ binding region and D143 in Helix1 were conserved.
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Keywords: Site-directed spin-labeling; Electron spin resonance; Prion; Conformational change; pH-sensitive region

The cellular prion protein (PrP^c) is a glycosylphosphatidylinositol (GPI)-plasma membrane-anchored protein whose function is still under debate [1–10].

Conversion of PrP^c from an α -helix- to a β -sheet-rich structure (the scrapie prion protein, PrP^{Sc}) causes relevant biophysical changes to the protein that have been related to brain dysfunction in prion diseases [1–3]. The mechanisms involved in the conversion are unknown. However, accumulating evidence suggests that

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