phisms of the human PrP gene have been found, the substitution of amino acids occurs only in the case of 1 repeat deletion (8 amino acids) in OR, M129V in HR1, N171S, and E219K aside an S-S bond. One repeat deletion and N171S are thought not to be related to the onset of and susceptibility to prion disease [23, 24]. On the other hand, the major polymorphism in the PrP gene (M129V) has been found to be related to susceptibility to prion diseases (Fig. **1A**). The most important point is that M129V strongly affects susceptibility to sporadic, iatrogenic, and variant CJD [25-27]. In addition, a polymorphism at codon 129 influences the phenotypes of prion diseases. This means that M129V is closely related to the clinical status and Western blotting patterns of PrP. Interestingly, E219K has not been still found in patients with sporadic CJD, suggesting a protective role for E219K against the onset of sporadic CJD [28]. Many mutations in the human PrP gene have been also found and known to be closely related to inherited prion diseases. Various phenotypes are observed when these mutations and the M129V polymorphism occur. The main phenotypes are GSS, which shows PrP plaques in the brain with a slow progression, familial CJD, which shows a CJD-like status, and FFI, which involves strong insomnia and neurodegeneration of thalamas. Interestingly, mutations related to prion diseases concentrate in the region between sites of disulfide bonds. In this review, we focus only on representative mutations related to GSS, FFI, and other familial CJDs. GSS is a group of syndromes showing inherited dementia with cerebellar abnormalities including amyloid plaques. The major GSS mutation is P102L(129M), which means P102L with M at the position 129. Patients with P102L(129M) show spongiform-related changes in the cerebrum and amyloid plaques in the cerebrum and cerebellum [29]. The P105L(129V) mutation, which means P105L with V at the position 129, is found in patients with spastic and paralytic GSS which has a less severe clinical onset than P102L-related GSS and does not show any spongiform in the cerebrum [30]. Y145STOP(129M), which means Y145STOP with M at the position 129, also causes GSS with Alzheimer's neurofibrillary tangles (NFTs) in the cerebral cortex and amyloid deposits around blood vessels [31]. D178N(129M) is associated with FFI, patients with which show no visible abnormalities or slight atrophy in the brain [32]. The most characteristic abnormalities are neurodegeneration and gliosis in the thalamus without spongiform and synaptic pattern of staining with anti-PrP antibody in subcortical gray matter and the brain stem, cerebellum, and inferior olivary nucleus. On the other hand, patients with D178N(129V), which means D178N with V at the position 129, show typical CJD with spongiform-related changes and synaptic PrP deposits and without amyloid deposits and neurodegeneration of thalami and the olivary nucleus [33]. As other mutations related to prion diseases, the deletion and insertion of repeats in the OR (-16, +8, 16, 32, 40, 48, 56, 64, 72), V180I, V180I-M232R, E200K, V203I, R208H, V210I, E211Q and M232R have been reported. All these deletions and insertions cause GSS or CJD with affected individuals showing spongiform, neurodenegeration, gliosis and amyloid deposits [34, 35]. Patients with V180I(129M/V) and V180I(M232R), which mean V180I with M or V at the position 129 and V180I with M→R at the position 232 respectively, show clinical symptoms of CJD such as spongiform change without amyloid deposits in various regions including the cerebral cortex and thalami [36]. There are many reports regarding the mutation E200K, patients with which show a typical CJD pathological status and additionally gliosis [37].

A number of bovine PrP gene polymorphisms have been also reported. The polymorphisms were found in two functionally important regions of the bovine PrP gene [5, 10, 38]. The first region consisted of the putative promoter region together with exons 1 and 2. The putative promoter region seems to encompass approximately 2.5 kb upstream of the transcription start site. The second region consisted of the entire third exon with the 795-bp ORF. To date, a total of 114 bovine PrP gene polymorphisms have been identified in different cattle breeds. Of these 114, 97 are single nucleotide polymorphisms (SNPs), 3 are polymorphisms involving two adjacent nucleotides, 3 are single base-insertion/deletions (Ins/Dels), 1 is a 2-base Ins/Del, 1 is a 23bp, 1 is a 12bp and 1 is a 14bp Ins/Del, and 1 is an OR polymorphism (Table **2**). Thirty eight of these polymorphisms were located in the putative promoter region, 17 in the ORF and 16 in exon 3. The majority of changes occur in the 5'-flanking region of the PrP gene rather than the ORF.

The 12bp and 23bp Ins/Del polymorphisms were found in all breeds investigated [5, 39-53]. In German cattle breeds, the allelic frequency of the 23bp insertion is around 0.43 in healthy cattle and 0.27 in BSE-affected cattle. The 23 bp insertion is more frequently found in healthy cattle. In Japan, its allelic frequency is 0.41 in healthy Japanese Black cattle breeds and 0.21 in healthy Holstein breeds.

Polymorphisms in the PrP gene ORF have been detected in many cattle breeds [38, 40, 42, 54-64]. Several breeds have the same polymorphisms. Interestingly, Japanese Indonesian cattle and Brazilian Caracu cattle have 12 of the same polymorphisms. Although, importantly, there are many polymorphisms in the bovine PrP gene ORF, no mutations have been associated with susceptibility to BSE [58, 60]. Very recently, a novel mutation, E211K, within the PrP gene was found to be associated with a case of H-type BSE [62-64]. Several polymorphisms in the bovine PrP gene promoter influence PrP expression (Fig. **1B**). Notably, an association of BSE-susceptibility with PrP gene genotypes at the 23 bp Ins/Del polymorphism in the 5' flanking region and the 12bp Ins/Del polymorphism within intron 1 of the bovine PrP gene was demonstrated in different cattle breeds [5, 39, 40, 42-53]. Electrophoretic mobility shift assays (EMSAs) have demonstrated that the 12 bp insertion allele was able to bind Sp1, and the 23bp insertion allele produced strong and specific band shifts with the transcription factor RP58. Also, reporter gene assays have demonstrated that the 23 bp insertion decreased PrP gene promoter activity. Furthermore, single polymorphisms in the promoter region also influence promoter activity [65]. In Japanese Black cattle breeds, there is a polymorphism at -6 bp of the promoter region, which is considered to be a Sp1-binding site. Reporter gene assays revealed that substitution of the Sp1-binding site at nucleotide -6 resulted in a significant decrease in activity compared with that in the nonsubstituted PrP gene promoter region. More recently, Xue *et al.* reported that Ins/Del polymorphisms in the upstream region and polymorphisms in the Sp1-binding site together affect promoter activity [51].

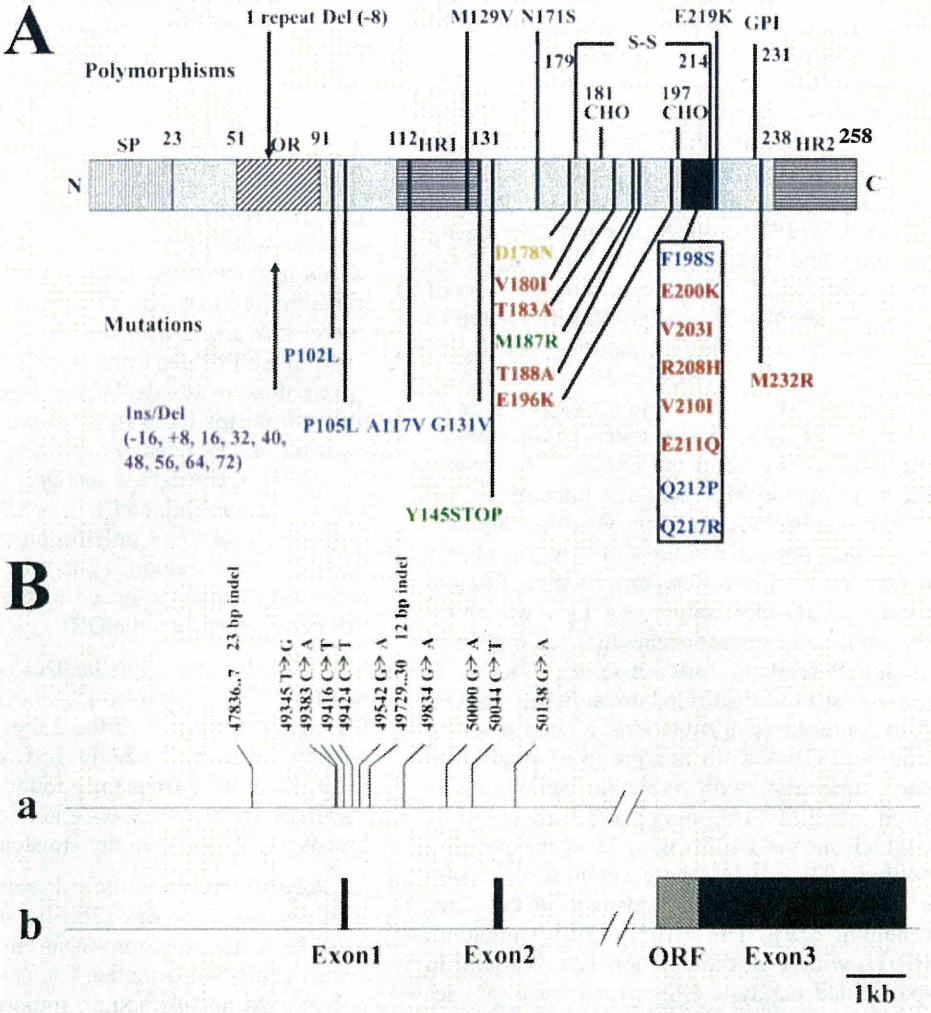

Fig. (1). Polymorphisms and mutations in the human and bovine PrP genes.

(A) The first 22 amino acid residues of the N-terminal of human PrP are cleaved shortly after translation, and the last 24 residues of the C-terminal are cleaved before the addition of the glycosylphosphatidylinositol (GPI) anchor. An octapeptide repeat region (OR) exists in the N-terminal. Polymorphisms (upper) and mutations (lower) of the human PrP gene are shown. GPI: glycosyl-phosphatidylinositol (GPI) anchor site; glycosylation site: CHO; SP: signal peptide region (SP); HR1: hydrophobic region in central part of PrP; HR2: hydrophobic region in C-terminal part of PrP; Red: mutations related to Creutzfeldt-Jakob disease (CJD); Blue: mutations related to Gerstmann-Sträussler-Scheinker syndrome (GSS); Purple : mutations related to GSS or CJD; Orange: mutations related to Fatal familial insomnia (FFI) or CJD; Green: mutations related to other diseases (Y145STOP: PrP cerebral amyloidosis; M187R: Inherited prion encephalopathy with curly PrP deposits). **(B)** Genomic structure of the bovine PrP gene. (Adopted from Sander *et al.*, 2004 [40]) and polymorphisms associated with PrP expression (Based on the results in Sander *et al.*, 2004; Nakamura *et al.*, 2007; Kashkevich *et al.*, 2007 [40, 45, 65]). Del: deletion; Ins/Del: insertion or deletion. M129V: Met→Val at the position 129; N171S: Asn→Ser at the position 171; E219K: Glu→Lys at the position 219; P102L: Pro→Leu at the position 102; P105L: Pro→Leu at the position 105; A117V: Ala→Val at the position 117; G131V: Gly→Val at the position 131; Y145STOP: Tyr→Stop codon at the position 145; D178N: Asp→Asn at the position 178; V180I: Val→Ile at the position 180; T183A: Thr→Ala at the position 183; M187R: Met→Arg at the position 187; T188A: Thr→Ala at the position 188; E196K: Glu→Lys at the position 196; F198S: Phe→Ser at the position 198; E200K: Glu→Lys at the position 200; V203I: Val→Ile at the position 203; R208H: Arg→His at the position 208; V210I: Val→Ile at the position 210; E211Q: Glu→Gln at the position 211; Q212P: Gln→Pro at the position 212; Q217R: Gln→Arg at the position 217; M232R: Met→Arg at the position 232.

The coordinated regulation of the bovine PrP gene promoter suggests that the Sp1 binding site polymorphisms control the binding of Sp1 to the PrP gene promoter and its activity. Taken together, from these observations, it may be possible to breed cattle with lower levels of PrP gene expression to reduce the risk of BSE. Although PrP gene-knockout cattle and sheep were already produced, there remains strong opposition to the use of genetically-modified food products such as plants and animals. Therefore, the use of cattle strains with lower PrP levels may provide an alternative approach to reducing prion risk. In this review, we avoid further commentary on polymorphisms and mutations of the PrP gene, as extensive reviews on the genetics of PrP in humans and ruminants including sheep, goats, and deer have been published [66, 67].

Table 2. Sequence Variants in the Bovine PrP gene

	Position	Variant	Breed	Genebank Accession Numbers	References
Promoter region	47004	A→G	German cattle	AF465161	[40]
	47221	C→T	German cattle	AF465162	[40]
	47238	C→T	German cattle	AF465163	[40]
	47450	C→T	German cattle	AJ298878	[40]
	47836..47837	23 bp Ins/Del	German cattle; Japanese Black cattle; UK Holstein; Swiss cattle; Aberdeen Angus, Charolais and Franqueiro; Bavi Village, Hatai Province and Vietnam cattle; Local Turkish cattle; Bos taurus, Bos indicus, and composite cattle; U. S. Cattle	AJ298878	[5, 40, 42-53]
			Swiss cattle,	AJ298878	[46]
	47854	A→G	German cattle	AJ298878	[40]
	47884	A→G	German cattle	AJ298878	[40]
	48004	C→G	German cattle	AJ298878	[40]
	48023	A→G	German cattle	AJ298878	[40]
	48129	C→T	German cattle	AJ298878	[40]
	48136	C→G	German cattle	AJ298878	[40]
	48161	A→C	German cattle	AJ298878	[40]
	48170	G→T	German cattle	AJ298878	[40]
	48194..48195	CG→GA	German cattle	AJ298878	[40]
	48429	A Del	German cattle	AJ298878	[40, 45]
	48476	C→T	German cattle	AJ298878	[40, 45]
	48524..48525	GG→C	German cattle	AJ298878	[40, 45]
	48567	C→T	German cattle	AJ298878	[40, 45]
	48584	C→A	German cattle	AJ298878	[40, 45]
	48689	C→T	German cattle	AJ298878	[40, 45]
	48695	G→T	German cattle	AJ298878	[40, 45]
	48700	G→A	German cattle	AJ298878	[45]
	48732	A→C	German cattle	AJ298878	[40, 45]
	48773	A→C	German cattle	AJ298878	[40]
	48815	A→C	German cattle	AJ298878	[40]
	48890	A→T	German cattle	AJ298878	[40]
	48921	C→T	German cattle	AJ298878	[40]
	49246	A→G	German cattle, Japanese Black cattle, Holstein-Friesian cow	AJ298878	[38, 40, 65]
	49289	T→C	Japanese Black cattle	AJ298878	[65]
	49345/721	T→G/ G→T	German cattle; Japanese Black cattle/ German cattle	AJ298878/D26150	[40, 45, 59, 65]
	49383	C→A	Japanese Black cattle	AJ298878	[65]
	49416/789	C→T/ T→C	German cattle	AJ298878/D26150	[45, 59]

(Table 1) contd....

	Position	Variant	Breed	Genebank Accession Numbers	References
	49424	C→T	Japanese Black cattle	AJ298878	[65]
	49446	C→T	Japanese Black cattle	AJ298878	[65]
	49474	C→T	Japanese Black cattle	AJ298878	[65]
	49493	G→T		AJ298878	[45]
	49542/ 914	G→A	Holstein-Friesian cow; German cattle	AJ298878/D26150	[38, 45, 59]
	49729..49730	12 bp Ins/Del	German cattle, Japanese Black cattle and Holstein cattle, UK Holstein, Swiss cattle, U. S. cattle, Aberdeen Angus, Charolais and Franqueiro; Bavi Village, Hatai Province and Vietnam cattle; Local Turkish cattle; Bos taurus, Bos indicus, and composite cattle; Holstein-Friesian cow	AJ298878	[5, 39-53]
	49834	G→A	German cattle	AJ298878	[40, 45]
	49871	A→G	German cattle	AJ298878	[45]
	50000	G→A	German cattle, Holstein-Friesian cow	AJ298878	[38, 40, 45]
	50044	C→T	German cattle	AJ298878	[38, 40, 45]
	50138	G→A	German cattle	AJ298878	[38, 40, 45]
	50297	G→A	German cattle, Holstein-Friesian cow	AJ298878	[38, 40, 45]
	50308	G→A	German cattle	AJ298878	[40, 45]
	50138	G→A	German cattle	AJ298878	[45]
	50319	G→A	German cattle, Holstein-Friesian cow	AJ298878	[38, 40, 45]
	50352	G →A	German cattle, Holstein-Friesian cow	AJ298878	[38, 40, 45]
	50376	G→A	German cattle	AJ298878	[38, 40, 45]
	50485..50486	CC→TT	German cattle	AJ298878	[40]
	50490	C→T	German cattle	AJ298878	[40]
	50518	T Del	German cattle	AJ298878	[40]
	50743	A→G	German cattle	AJ298878	[40]
	51189	T Del	German cattle	AJ298878	[40]
	51199	G→A	German cattle, Holstein-Friesian cow	AJ298878	[38, 40]
	51208	C→T	German cattle	AJ298878	[40]
	52183	G→A	Holstein-Friesian cow	AJ298878	[38]
	52445	C→A	Holstein-Friesian cow	AJ298879	[38]
	52587	G→A	Holstein-Friesian cow	AJ298879	[38]
	52793	T→ C	Holstein-Friesian cow	AJ298879	[38]
	54863	TTGTACATT→ GTAACTTTGTT CTCCATTTCTT TTATGATATAT GGTCAAGTTTT CTATTTAAAA ATTG	Holstein-Friesian cow	AJ298879	[38]
	55003	T→C	Holstein-Friesian cow	AJ298879	[38]

(Table 1) contd....

	Position	Variant	Breed	Genebank Accession Numbers	References
	55171	G→A	Holstein-Friesian cow	AJ298879	[38]
	55196	A→G	Holstein-Friesian cow	AJ298879	[38]
	55339	T→A	Holstein-Friesian cow	AJ298879	[38]
	55830	G→A	Holstein-Friesian cow	AJ298879	[38]
	55903	CAC→GAACT TGTTA	Holstein-Friesian cow	AJ298879	[38]
	57904	A→G	Holstein-Friesian cow	AJ298879	[38]
	57991	CGA Del	Holstein-Friesian cow	AJ298879	[38]
	58212	T→A	Holstein-Friesian cow	AJ298879	[38]
	58999	G→A	Holstein-Friesian cow	AJ298879	[38]
	59201	G→C	Holstein-Friesian cow	AJ298879	[38]
	60001	(A)5→(A)7	Holstein-Friesian cow	AJ298879	[38]
	60291	T→C	Holstein-Friesian cow	AJ298879	[38]
	60835	A→ G	Holstein-Friesian cow	AJ298879	[38]
	61326	A→G	Holstein-Friesian cow	AJ298879	[38]
	62413	G→A	Holstein-Friesian cow	AJ298879	[38]
	62486	G→A	Holstein-Friesian cow	AJ298879	[38]
	63097	G→T	Holstein-Friesian cow	AJ298879	[38]
	63257	G→T	Holstein-Friesian cow	AJ298879	[38]
	65165	A→G	Holstein-Friesian cow	AJ298879	[38]
ORF	65647	C→T	Japanese Indonesian cattle; Brazilian Cararu cattle; Britainic cattle	AJ298878	[58, 60, 61]
	65653	G→A	Japanese Indonesian cattle; Brazilian Caracu cattle	AJ298878	[58, 60]
	65686	T→G	Japanese Indonesian cattle; Brazilian Caracu cattle	AJ298878	[58, 60]
	65704	A→G	Japanese Indonesian cattle; Brazilian Caracu cattle	AJ298878	[58, 60]
	65802..65825	24 bp octapeptide repeat	German cattle, Japanese Black cattle and Hostein cattlecattle, Brazilian Caracu cattle, Korean Holstein cattle, U.S. cattle	AJ298878	[38, 40, 42, 54-58]
	65812	G→A	German cattle; Japanese Indonesian cattle; Japanese Bos taurus cattle; Brazilian Caracu cattle; British cattle; Holstein-Friesian cow; Korean Hanwoo and Holstein cattle	AJ298878/X55882	[38, 40, 57-60]
	65917	C→T	German cattle; Japanese Indonesian cattle; Japanese Bos taurus cattle, Japanese Black cattle and Holstein cattle; Brazilian Caracu cattle; British cattle	AJ298878/X55882	[40, 42, 58-61]
	66039	G→A	Japanese Indonesian cattle; Brazilian Cararu cattle; Britainic cattle	AJ298878	[58, 60, 61]
	66132	A→G	Japnanese Indonesian cattle	AJ298878	[60]
	66133	C→T	Japanese Indonesian cattle; Brazilian Caracu cattle	AJ298878	[58, 60]
	66154	C→T	German cattle; Japanese Bos taurus cattle;Japanese Black cattle and Holstein cattle; Brazilian Caracu cattle; British cattle; Holstein-Friesian cow; Korean Hanwoo and Holstein cattle	AJ298878/X55882	[38, 40, 42, 58-61]

(Table 1) contd....

	Position	Variant	Breed	Genebank Accession Numbers	References
	66208	C→T	Japanese Indonesian cattle; Brazilian Caracu cattle	AJ298878	[58, 60]
	66209 E211K	GAA→AAA (E→K)	U.S. cattle	AY335912	[62-64]
	66253	C→T	Japanese Indonesian cattle	AJ298878	[58, 60]
	66256	T→C	Japanese Indonesian cattle; Japanese Black cattle and Holstein cattle; Brazilian Caracu cattle	AJ298878	[42, 58, 60]
	66361	C→T	Japanese Indonesian cattle; Japanese Black cattle and Holstein cattle	AJ298878	[58, 60]
	66877	C→T	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
3' untranslated region	66906	A→G	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
	66948	C→T	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
	67477	G→T	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
	67490..67491	AG Del	German cattle	AJ298878	[40]
	67598	A→G	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
	67864	A→G	German cattle; Holstein-Friesian cow	AJ298878	[38, 40]
	68019..68046	14 bp Ins	German cattle; U. S. cattle; Holstein-Friesian cow	AJ298878	[38, 40, 41]
	68408	del AAGAA	Holstein-Friesian cow	AJ298878	[38]
	68539	T→A	Holstein-Friesian cow	AJ298878	[38]
	68548	A→T	German cattle	AJ298878	[40]
	68620	T→G	Holstein-Friesian cow	AJ298878	[38]
	68652	C→T	German cattle	AJ298878	[40]
	69085	A→G	German cattle	AJ298878	[40]
	69660	T→G	Holstein-Friesian cow	AJ298878	[38]
	69684	(T)4→(T)5	Holstein-Friesian cow	AJ298878	[38]
	69774	G→A	Holstein-Friesian cow	AJ298878	[38]

Polymorphisms affecting susceptibility to BSE prion infection are written in red.
Polymorphisms affecting PrP expression are written in blue.
Ins: insertion; Del: deletion; Ins/Del: insertion or deletion.
E211K: Glu→Lys at the position 211.

Structure and Functions of PrP Determined by Experimental Biochemical and Biophysical Approaches

PrP contains OR [P(Q/H)GGG(G/-)WGQ] toward the N-terminal end that has affinity for divalent metals, such as Cu, Zn, Ni, and Mn, with a preference for binding Cu [68, 69]. Therefore, PrP^C is thought to possess a Cu-dependent enzyme function, specifically that of an antioxidant activity, like superoxide dismutase (SOD) [70], or participate in Cu homeostasis [71]. However, certain conditions such as refolding in the presence of a high concentration of Cu appear to be required for its SOD-like activity because purified recombinant PrPs from insect and mammalian cells did not show any SOD-like activity [72, 73]. Endocytosis of PrP^C is promoted at high Cu concentrations [74]. In fact, PrP^C constructs lacking the OR failed to undergo endocytosis efficiently [74], exhibiting significant superoxide dismutase (SOD)-like activity [70]. In addition, PrP^C regulates cellular anti-oxidative activity including Cu/Zn SOD [75], glutathione (GSH) [71], glutathione reductase (GR) [71, 76], catalase [71, 77], and ornithine decarboxylase [77]. Therefore, PrP^C plays a crucial role in Cu metabolism and anti-oxidative defense.

At present, over 70 PrP structures are available from the Protein Data Bank (PDB). About 50 nuclear magnetic resonance (NMR) structures and about 20 X-ray crystallographic structuresof PrP^C are registered in the bank. All are PrP^C structures. A proposed structure for human PrP^C is shown in Fig. (**2**). Human PrP^C is composed of a highly structured C-

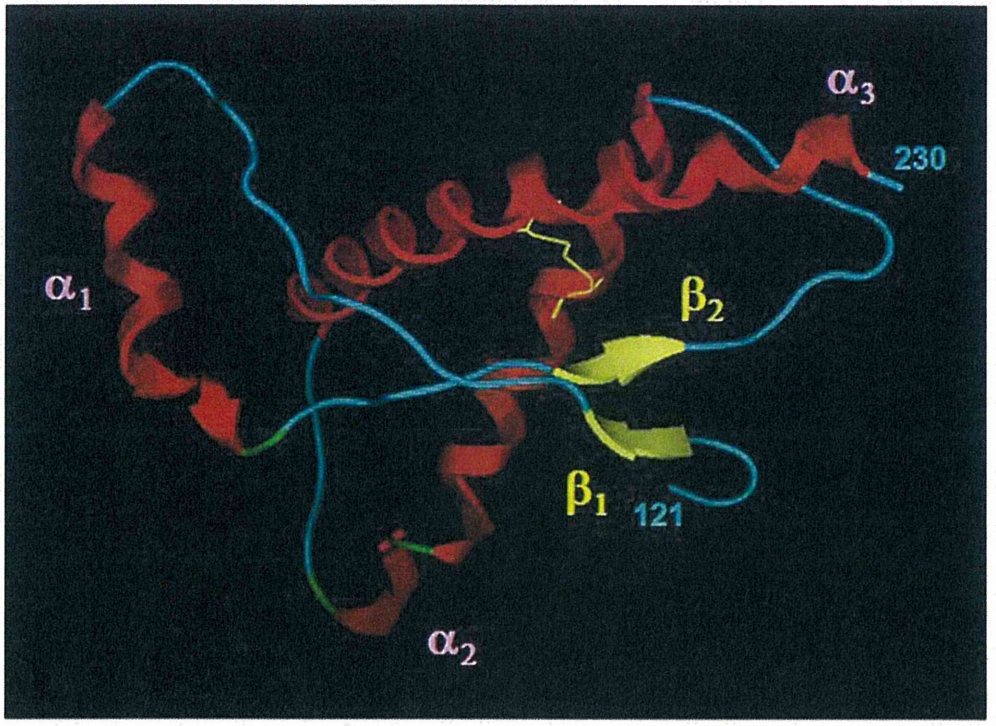

Fig. (2). Ribbon diagram of the human PrP structure.
The C-terminal domain of PrP (amino acid residues 121-230), which is highly structured is shown. The 3 α-helices (Red allows) and 2 β-sheets (Yellow allows) are indicated. Helix α1 comprises residues D144–H155; helix α2, residues N173-K194; and helix α3, E200-Q227. The β1 sheet comprises Y128-G131, and the sheet β2 comprises V161-R165. There are 3 α-helices and 2 β-sheets in the C-terminal domain. Two of the 3 α-helices, α2 and α3, are linked by a disulfide bond. The yellow bond represents the disulfide bond between C179 and C214. Green, blue, and aqua lines show the turn of 4-5 amino acid residues, turn of 3 amino acid residues, and random coil, respectively. A ribbon diagram of human PrP obtained from 1QLX in the protein data bank [79] is shown. The illustration was generated with the Molecular Operating Environment modeling package (MOE, Chemical Computing Group, Inc., Montreal, Canada). D144: Asp at the position 144; H155: His at the position 155; N173: Asn at the position 173; K194: Lys at the position 194; E200: Glu at the position 200; Q227: Gln at the position 227; Y128: Tyr at the position 128; G131: Gly at the position 131; V161: Val at the position 161; R165: Arg at the position 165; C179: Cys at the position 179; C214: Cys at the position 214.

terminal region (residues 121–232) that has 3 α-helices and double-stranded antiparallel β-sheets (roughly 110 residues) and an unstructured N-terminal tail (spanning the first 120 residues approximately) [78-83]. The NMR structure of the N-terminal domain including OR was not determined due to its high flexibility, but possibly the region is unfolded. Recent studies have shown that the structured C-terminal region of PrP can also bind Cu ions [84-86].

Hypothetical Mechanism of PrP^{Sc} Conversion Based on Experimental Approaches

The conformational change from PrP^{C} to PrP^{Sc} is crucial to the pathogenicity of the prion. Once PrP^{Sc} is generated, it acts as a template for the conversion of endogenously expressed PrP^{C} to PrP^{Sc}. PrP^{Sc} is thought to be formed by a self-replication mechanism [87, 88]. The conformational change from PrP^{C} to PrP^{Sc} induces a dramatic change in secondary structure. Infrared spectroscopy has shown that the percentage of β-sheets increases but that of α-helices decreases during the change from PrP^{C} to PrP^{Sc} [89]. The high β-sheet content allows PrP^{Sc} to aggregate into insoluble fibrils and acquire proteinase K (PK)-resistance [87].

There are several models that explain the conversion of PrP^{C} to PrP^{Sc}: refolding and seeding models. The former proposes that a single particle of PrP^{C} interacts with PrP^{Sc} to form more PrP^{Sc} [90]. The latter model predicts that an oligomer or short polymer of PrP^{Sc}, instead of single PrP^{Sc} particles, interacts with PrP^{C} to form more PrP^{Sc} particles [91, 92]. There has been some speculation about the requirement of an additional chaperone protein, namely, protein X, for propagation, while other models have maintained that an imbalance in metal binding may be a key factor in the formation and propagation of PrP^{Sc} [93]. However, these hypotheses have not yet been proved. As PrP^{Sc} is difficult to crystallize, it can not be subjected to X-ray crystallography. In addition, as PrP^{Sc} is insoluble in water, it can not be analyzed by NMR spectroscopy. If PrP^{Sc} is solubilized using detergents and sonication, its infectivity can decrease. These problems prevent a structural analysis of PrP^{Sc} experimentally. Recent studies using computational analyses have provided a clue as to the structure of PrP^{C} and PrP^{Sc} and mechanism of conversion from PrP^{C} to PrP^{Sc}. Next, we will introduce recent reports on the potential structure of PrP^{C} and PrP^{Sc} revealed by computational analysis such as molecular dynamics (MD) and *ab initio* calculation.

Computational Simulation Analysis of PrP Structures and their Conversion

From experimental approaches, the structures and functions of PrP^C have been studied. PrP is a Cu-binding protein as mentioned above. The binding is thought to involve the octapeptide repeat units in the N-terminal region [94]. Recent experiments have shown that Cu(II) also binds to the structured C-terminal [84-86]. The histidine residue has an important role to play in Cu(II) binding [95]. Some Cu(II)-binding sites were located in the region around H96 and H123 in the N-terminal domain and in the region around H140, H177, and H187 in the C-terminal domain in human PrP (residues 91–231) [96]. Recently, scientists have been trying to study these issues using computational analyses.

In these Cu-binding systems, *ab initio* calculations, such as molecular orbital calculations, are suitable, because they are precise. This is a particularly important method of calculation in the case of systems dealing with the movement of electrons, such as chemical reactions, and systems dealing with transition metals since MD can not be applied to these systems. Zidar *et al.* calculated the location of 3 Cu(II)-binding sites (H140, H177, and H187) in the C-terminal portion of PrP^C by *ab initio* quantum mechanics (QM)/molecular mechanics (MM) simulation [96]. The results indicated that H140 did not bind to Cu(II) since the calculation did not converge, and H177 might not bind to Cu(II) because a neighboring Asp bound to Cu(II) in its place. However, they concluded that H187 can bind to Cu(II) since the distance between 1 of the 2 nitrogen atoms (NE1) of H187 and Cu(II) approached each other from the start position to the end position in most of their calculations. More importantly, they proposed that the binding of Cu(II) to His residues in the C-terminal portion of PrP^C may be involved in the conversion of PrP^C to PrP^{Sc}. Ji *et al.* used the density functional theory (DFT) method B3LYP/LANL2DZ to confirm the most plausible Cu(II)-binding mode of human PrP^C [97]. They calculated the electron affinity (EA), which is the theoretical index for characterization of the electron transfer rate, and proposed binding mode IV, the most probable mode, where human PrP^C had a higher EA than that of existing antioxidants such as SOD. Meanwhile, Cu(II)-binding mode IV consists of four equatorial atoms and one axial atom. The four equatorial positions are occupied by one amide nitrogen and one carboxyl oxygen in one glycine, one amide nitrogen in the other glycine, and δ-nitrogen in histidine. The axial position is occupied by water oxygen. Finally, as the molecule has a higher electron transfer rate and a lower EA value, they concluded that PrP^C is a Cu(II) transporter rather than an antioxidant.

In the study of metal-binding proteins such as PrP, the accuracy of the potential used can cause problems when classical MD simulation is used for calculations. To improve the accuracy of the potential, a novel method (*ab initio* MD) has been recently developed. Since this method involves the use of the potential calculated by QM, it has proven to be more accurate than classical MD simulation; however, it should be noted that it cannot be used to treat a large number of atoms at different time intervals. For example, the Car-Parrinello method cannot be applied to this method [98]. Recently, several scientists have examined PrP using *ab initio* MD simulation. Furlan *et al.* investigated the Cu(II) coordination around binding sites of the PrP OR using 1.4–2.4 ps *ab initio* MD [99]. They carried out simulations in the following systems: the Cu(HG$^-$G$^-$GW)(wat) complex, Cu(HG$^-$G$^-$G), Cu(HGGG) with a number of water molecules and with protonated Gly2 and Gly3, and the $[Cu(HG^-G^-G)]_2$ dimer. They showed that binding between Cu and the amide nitrogen of the deprotonated glycine residues is very stable in all cases despite the presence of a Trp residue and water.

In addition, Colombo *et al.* reported a 13 ps QM/MM MD simulation in mouse PrP at 6 binding sites of the C-terminal domain [100]. The results showed that 4 of the 6 binding sites, namely, H140_M [Cu(II)-binding sites included H140 and M138], H140_DD [Cu(II)-binding sites included D144 and D147 and not a His residue], H177, and H187_E [Cu(II)-binding sites included H187 and E197] possibly existed; however, H140_D [Cu(II)-binding sites included H140 and D144] and H187_D [Cu(II)-binding sites included H187 and D202] were not consistent with the experimental results [101].

Computational simulation has also contributed to better understanding of prion diseases. An MD simulation of human PrP (90–230 residues) was performed by homology modeling with Syrian hamster PrP(90–231) [102]. This was the first report on the potential structure of human PrP; it was obtained by carrying out 600–1500 ps MD simulations in water. In their system, the correct treatment of electrostatic interactions was very important. Ions such as Na^+ and Cl^- stabilized the whole system, especially the α-helix, but divalent cations did not result in further stabilization. Moreover, they showed that simulation with the particle mesh Ewald (PME) method was effective in these systems because an 8 Å cutoff during simulation resulted in loss of the secondary structure. Furthermore, a stable main core (helices B and C) is linked to more flexible structured parts (helix A and strands A and B) by three salt bridges such as (E146/D144 ↔ R208, R164 ↔ D178 and R156 ↔ E196) and contributes to PrP^C stability. More importantly, two of these salt bridges are associated with mutations in sporadic CJD (R208H) [103] and FFI (D178N) [104]. Therefore, the reports provided the first evidence for the importance of salt bridges to the stability of PrP and further, the pathogenesis of inherited prion diseases.

By contrast, other groups reported results inconsistent with the above findings. Gsponer *et al.* reported a 1.5 ns MD simulation of a D178N mutant of mouse PrP(124–226) in water molecules using a spherical boundary [105]. The main purpose of the study was to examine the structural rearrangement and increase in flexibility of PrP caused by the D178N mutation. Unfortunately, the mutant model did not reveal any major structural rearrangement, but they showed that if the simulation was carried out with a 12 Å cutoff, the secondary structure was not lost. Furthermore, they suggested that the salt bridge between R164 and D178 might not be associated with the stability of PrP. In addition, Bamdad *et al.* carried out a 10 ns MD simulation of an R208H mutant of the human PrP C-terminal domain (residues 115–228) to re-evaluate the contribution of a disease-associated salt bridge [106]. The simulation was performed in water and with a periodic boundary at 300 K. They then compared their

result with the reported structure obtained by NMR spectroscopy and protonated forms of D144/E146 whose residues formed a salt bridge between R208. The mutant model showed a higher root-mean-square deviation (RMSD) than the wild type and protonated structures; further, it showed structural instability, especially around residues 152–156 (at the end of helix α1) and 193–196 (helix α2). The R208H mutation resulted in the removal of a hydrogen bond between H208 and K204, which is the N-terminal end of helix α3, and the mutant model showed large structural changes.

Recently, Guilbert *et al.* carried out a 1.75 ns MD simulation of mouse PrP(121–231) [107]. The simulation was performed in water molecules with a periodic boundary conditions at 300 K. They then compared the result with the NMR structure. Interestingly, they found a novel β-sheet in a hydrophobic cluster after 70–100 ps. The new sheet was formed as a result of 3 new hydrogen bonds between residues 123–125 and 128–130. During the simulations, the Ψ and Φ values of the simulation and experimental structures were in agreement in the case of residues 127–131 and 161–163, which were regions forming a β-sheet. However, the region 124–127 showed some differences due to the formation of a third strand. The folding mechanism of the third β-sheet was followed. First, the hydrogen bond between G123 and L130 was formed. Then, hydrogen bonds between G124 and M129, L125, and Y128 were formed after rotation of Ψ125 and Φ125. Importantly, the result may provide insights into the conversion of PrP^{C} to PrP^{Sc}.

All of these MD simulations were of monomeric PrP. Sekijima *et al.* demonstrated a 10 ns MD simulation of human PrP [108]. This was the first simulation of the PrP^{C} dimer. They performed the simulations at 300 K and 500 K in a D178N model and an acidic pH model. They showed that denaturation of the helices and β-sheet elongation were common to both the monomer and dimer, but the monomer began denaturing before the dimer. These results indicate the association of α-helix denaturation and β-sheet elongation with the structural stability of the monomer and dimer, suggesting that conformational changes of PrP^{C} occur together with dimerization.

Very recently, MD simulation has provided a novel idea as to the mechanism of conversion from PrP^{C} to PrP^{Sc}. The model, where the most stable PrP^{Sc} unit is considered to be a hexamer, which plays an important role as the minimum infectious unit, seems to be suitable compared to seeding and refolding models based on MD simulations [109]. In the model, PrP^{C} attaches to the PrP^{Sc} template, the PrP^{Sc} oligomer, which is converted to the PrP^{Sc} trimer. The resultant rod-like PrP^{Sc} is broken and acts as template for the conversion.

All in all, computational simulation has the potential to provide useful information on prion diseases and the physiological functions of PrP. With the further development of calculation methods and speeds, computational simulation would become more and more important for addressing the issue of prion diseases.

CONCLUSIONS AND PERSPECTIVES

Prion diseases are zoonotic infectious diseases transmissible among animals and humans. The BSE prion is a cause of variant CJD in humans, and can be transmitted *via* blood. In addition, the risk of inherited and sporadic prion diseases and iatrogenic infection of prion remains, because prion diseases can not be cured due to an absence of therapy and drugs, even if preventative methods are established.

Using basic computational analyses of DNA sequences and amino acids, several mutations and polymorphisms in the PrP gene have been found. Perhaps, the most important finding of these analyses is the presence of mutations and polymorphisms directly related to inherited prion diseases and affecting susceptibility to sporadic, iatrogenic, and variant CJD. In addition, certain polymorphisms such as Sp1-binding site polymorphisms in the bovine PrP gene promoter region influence the promoter activity of the PrP gene, suggesting that breeding cattle with such substitutions may be a useful way to reduce the risk of BSE without the social resistance of consumers to genetically-modified food products such as PrP gene-knockout cattle.

In this review, we also introduced studies on PrP using computational methods such as MD simulations and *ab initio* calculations. These molecular simulations are very effective methods of resolving the molecular properties of proteins with abnormal properties like PrP, which is difficult to analyze by experimental methods. This method is expensive in terms of the time required by the CPU for calculations; however, with the increase in CPU power and development of multicore processors and novel calculation methods, such as the QM/MM (combined QM and molecular mechanics) [110, 111], Own N-layer Integrated molecular Orbital molecular Mechanics (ONIOM) method [112], fragment molecular orbital (FMO) method [113], etc, we can further analyze and carry out calculations even for an entire protein. There have not been many reports of computational analyses in this field, but further growth in this field, especially the application of these methods to PrP research, can be expected.

Finally, we wish to emphasize that computational analysis is also an important factor in the multivariate analysis of the exhaustive expression of proteins, genes, and carbohydrate chains. Recent developments in these fields, viz: proteomics, genomics, glycomics, and metabolomics, etc. will lead to more contributions of computational analysis.

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Infectious prion protein in the filtrate even after 15nm filtration

Mikihiro Yunoki [a,c,*], Hiroyuki Tanaka [a], Takeru Urayama [a,b], Yuta Kanai [b,1], Aya Nishida [a], Maki Yoshikawa [a], Yuji Ohkubo [a], Yoshiyasu Kawabata [a], Katsuro Hagiwara [b], Kazuyoshi Ikuta [c]

[a] Research & Development Division, Benesis Corporation, Japan
[b] School of Veterinary Medicine, Rakuno Gakuen University, Japan
[c] Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan

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ABSTRACT

The evaluation of the removal efficacy during manufacturing is important for the risk assessment of plasma products with respect to possible contamination by infectious prions, as recently reported in several papers on the potential for prion transmission through plasma products. Here, we evaluated a virus removal filter which has 15 nm pores. An antithrombin sample immediately prior to nano-filtration was spiked with prion material prepared in two different ways. The removal (log reduction factor) of prion infectivity using animal bioassays was ≥4.72 and 4.00 in two independent filtrations. However, infectivity was detected in both the pellet and supernatant following ultracentrifugation of the 15 nm filtered samples, indicating difficulty in complete removal. The data supports the conclusion that a certain amount of infectious prion protein is present as a smaller and/or soluble form (less than ~15 nm in diameter).

1. Introduction

Although the risk of transmission of classical or sporadic Creutzfeldt-Jakob disease (sCJD) through blood transfusion is theoretically possible, no verifiable case of transmission has been reported. However, the risk of contracting variant CJD (vCJD) through blood transfusion has been of increasing concern, particularly since the report of a fourth possible transmission case [1,2]. In addition, two investigations of cases involving recipients of plasma products manufactured from pooled source plasma containing a vCJD-infected donor were recently reported. In the first of these reports, abnormal prion protein was detected in a patient without symptoms of vCJD, revealed vCJD abnormal prion protein at post mortem in the patient (a haemophiliac) who had been treated with a Factor VIII product derived from a source material containing plasma that included a donor who developed vCJD after the donation. The UK Health Protection Agency retained their position of 'at risk' for UK derived plasma products [3]. The FDA considers 'the estimated risk' is highly uncertain but is most likely to be extremely small in the case of US-licensed plasma products [4]. A follow-up review of the case reported that the patient was more likely to have been infected by potential subclinical vCJD donors present in normal donor plasma, than by smaller quantities of plasma derived from the donor who had developed vCJD [5,6]. In another report, vCJD abnormal prion protein was not found in a post mortem examination of a patient with common variable immunodeficiency (CVID) who had been treated with an intravenous immunoglobulin (IVIG) product derived from a source material containing plasma from a donor who later developed vCJD, post mortem without symptoms of vCJD [7].

Experimental studies in animal models have demonstrated the transmission of bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), scrapie, CJD and vCJD through transfusion [2]. Furthermore, infectivity was detected in plasma derived from vCJD-infected mice [8]. To reduce the risk of transmission through biologics derived from raw materials potentially contaminated with infectious prion protein, such as plasma, safety measures against pathogen contamination should be employed. Such measures include decreasing the potential prion load, evaluating the risk of the product and employing prion removal step(s) in the manufacturing process wherever possible [9–11].

Nano-filtration has been reported as a very effective tool for the removal of prions [12–14]. These reports suggested that the biological properties of infectious prions in the spiking material could affect

* Corresponding author. Osaka Research Laboratory, Research & Development Division, Benesis Corporation, 3-16-89, Kashima, Yodogawa-ku, Osaka 532 8505, Japan. Tel.: +81 6 6300 2458; fax: +81 6 6300 2404.
E-mail address: Yunoki.Mikihiro@mk.mt-pharma.co.jp (M. Yunoki).
[1] Current address: Thailand-Japan Research Collaboration Centre on Emerging and Re-emerging Infections (RCC-ERI), Thailand

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evaluations of clearance. Foster [15] also reviewed the significance of the method of preparing the spiking material for clearance studies but further research is required due to a lack of consensus. Although infectious activity peaks markedly at 17–27 nm [16], our recent study reveals that even a 15 nm filter could not remove all infectious prion [14]. Our objective in this study is to clarify the infectivity of prion protein that penetrated the 15 nm filter.

2. Materials and methods

2.1. Quantitative removal capacity of 15 nm filter

To evaluate the quantitative removal capacity of a 15 ± 2 nm virus removal filter virus removal filter (Pnanova 15 N, 0.001 m^2, (P-15 N), Asahi Kasei Medical Co., Ltd. Tokyo, Japan) we used a sample of the antithrombin preparation (Neuart®, Benesis Corp., Osaka, Japan) taken immediately before the P-15 N step. Briefly, the microsomal fraction as a spiking material was prepared as follows. Brain homogenates from hamster adopted scrapie 263 K strain infected hamsters in PBS (10% w/v) were centrifuged at low speed (1,000 g for 20 min, at 4 °C) and the supernatant was treated with the 0.1% detergent lysolecithin (37 °C for 30 min). Then the homogenate was centrifuged at high speed (9,100 g for 10 min at 4 °C) and the supernatant was extensively sonicated on full power (20 kHz, 550 W, Misonix XL2020, Qsonica LLC., USA) for 5 min with 2 min intervals every 1 min sonication (5 ml/tube without cymbal rod). The homogenate obtained was sequentially filtered using 0.45, 0.22 and 0.1 μm filters was used as a spiking material. The starting material was spiked (1:50 v/v) and then filtered using P-15 N. The samples, before and after the filtration of two independent runs were titrated to determine the reduction of the PrP^{res} by Western blotting (WB2 method in reference 14). An animal bioassay (BA) was also performed to determine the reduction in infectivity. For the BA, four to five-week old specific pathogen free and viral antibody-free male Syrian hamsters were inoculated i.c. with 0.05 ml/animal of the ten-fold serially diluted sample. Six animals were used for each diluted sample. The animals were monitored for general health and clinical signs, and euthanized once advanced clinical signs were evident or at the end of the assay period (383 days). A histopathological analysis was performed on all brains from animals sacrificed in the study and log reduction factors were calculated following titre determinations by the method of Kärber. This investigational TSE clearance study was performed in accordance with GLP and guidances at BioReliance, Glasgow UK and Rockville US facilities [10,17,18].

2.2. Property of P-15 N-filtered samples

To determine the characteristics of prion infectivity in the filtrate, an analysis of filtrates from additional spiked runs was performed by ultracentrifugation and qualitative (200 days) infectivity assay. Microsomal fraction as spiking material was prepared as described in 2.1 (without detergent treatment) following ultracentrifugation to purify the microsomal fraction. The microsomal fraction was then extensively sonicated at 20 kHz, 200 W (Bioruptor UCD-200 T, Cosmobio Co., Ltd., Japan), 10 min with 1 min intervals every 1 min sonication (2 ml/tube with cymbal rod) and subsequently filtered using 0.22 μm filters. This filtrate was used to spike samples. The spiked (1:20 v/v) antithrombin samples were passed through a 15 nm filter. The resultant log reduction factor by Western blotting was ≥2.8 and infectivity was detected in the filtered sample [14]. The filtered sample was ultracentrifuged at 150000 g for 60 min at 4 °C and the pellet was resuspended with PBS. The resuspended pellet and supernatant were inoculated i.c. to three female-specific pathogen-free Syrian Hamsters with 0.02 ml/animal of these undiluted samples. As a control, a non-ultracentrifuged filtrate sample was also inoculated. The animals were euthanized once advanced clinical signs were evident or at the end of the assay period (200 days). A histopathological analysis of the brain from all sacrificed animals was also performed described as previous study [14].

3. Results

3.1. Capacity of the 15 nm filter to remove prion

The capacity to remove prions from the antithrombin preparations during Planova 15 N filtration using either extensively sonicated lysolecithin treated prions or extensively sonicated microsomal fractions are summarized in Table 1. The log reduction factors (LRFs) using the lysolecithin spike in the animal experiments were ≥4.72 and 4.00, respectively for the duplicate runs. These results revealed that the Planova 15 N filtration is "effective but not complete" for the removal of infectious prion contamination. One of the experiments showed that a small amount of infectious prion was still detectable in the filtrate. These results demonstrate that even 15 nm filtration may not be able to completely remove infectious prion (Table 1).

3.2. Qualitative removal capacity of 15 nm filter and subsequent analysis of the filtered sample

To clarify the properties of the infectious prion, the pellet and supernatant derived from the 15 nm filtrate (using a sonicated microsomal spike material) after ultracentrifugation were investigated. PrP^{res} was not detected by Western blot assay either in the filtrate, or in the supernatant and pellet by ultracentrifugation of the filtrate. In contrast, infectivity was detected in all samples by animal bioassay, a more sensitive assay method (Table 1). This result showed that a certain amount of infectious prion was able to penetrate the 15 nm virus removal filter and was not pelleted by ultracentrifugation. Of note, one of two animals which were inoculated with the supernatant showed slightly faster disease progression than other animals after the appearance of clinical signs in the study. However, histopathological observations did not show any clear differences between the supernatant and pellet fractions after ultracentrifugation.

4. Discussion

Clarification as to the real form of infectious prion protein in infectious human and animal plasma is very important in order to

Table 1
Scrapie PrP^{Res} and infectivity in samples generated with 15 nm filtration and subsequent ultracentrifugation

	Quantitative		Qualitative	
	WB	BA	WB	BA
Spiking material	lysolecithin treated and Extensively sonicated		Extensively sonicated	
Before filtration	6.1 / 6.1	7.97 / 8.30	3.6	+ve[c]
After filtration	<2.6 / <2.6	<3.25 / 4.30	<0.8	+ve[d]
Log reduction	≥3.5 / ≥3.5	≥4.72 / 4.00	≥2.8	NA
Pellet[a]	NA	NA	<1.0	+ve[e]
Supernatant[b]	NA	NA	<1.0	+ve[f]

+ve, scrapie positive. NA, not applicable.
[a] :Pellet fraction of ultracentrifuged filtrate.
[b] Supernatant fraction of ultracentrifuged filtrate.
[c] Clinical sign was observed from 90 ~ 118 days post infection.
[d] Clinical sign was observed from 111 ~ 175 days post infection.
[e] Clinical sign was observed from 111 ~ 113 days post infection.
[f] Clinical sign was observed from 125 ~ 175 days post infection.

evaluate the risks of prion contamination in plasma products and biopharmaceutical medicines. Some results suggesting the form of infectious prion protein in human and animal plasma have been reported. A genetically-modified animal plasma containing GPI-anchor less prion protein had some infectivity [19,20]. On the other hand, a high titer of prion remained in the supernatant of an ultracentrifuged microsomal fraction derived from scrapie-infected brain, although PrP^{res} was not detected by Western blot assay [21]. Although these results were obtained under experimental conditions, it suggests that the infectious prion protein may exist in animal plasma as a soluble or soluble-like form. Ultracentrifugation has been commonly used for the concentration of the prion protein. The ultracentrifugation and subsequent preparation of the spiking material should be done carefully in order to ensure that such preparations do not exclude such soluble-like prion protein. To avoid over estimating removal, pelleting of the spike by ultracentrifugation should not be used. However, preparation methods or employing treatment which generate small size of infectious prion such as sonication and/or detergent treatment following the ultracentrifugation (as performed in this study) should be used. Many studies to evaluate prion removal during manufacturing have been performed, however studies of the appropriateness of the spiking materials derived from prion-infected brain are limited. We reported that extensively-sonication and/or treatment with a detergent such as sarkosyl and lysolecithin were useful for the preparation of spiking material for analyzing particle size [14]. Hence, preparation methods without pelleting the prion by ultracentrifugation or with the treatment which generates soluble-like prion in the supernatant following ultracentrifugation will lead to more acceptable results for the evaluation of TSE removal, especially when an animal study is included.

In this study, we evaluated the prion removal performance of nano-filtration on a lab scale using a 15 nm Planova filter and a sample of antithrombin which was spiked with infectious prion protein. Two types of spiking material were used. Both spiking materials used in this study seemed to contain soluble-like infectious prion protein because of the preparation methods employed sonication treatment which seems to generate the soluble-like form infectious prion. Hence, the results of the filtrate sample and LRF in the studies can be considered realistic for evaluation of the filtering process with respect to prion removal.

Residual infectivity was detected in the filtered process sample of antithrombin preparations which was spiked with extensively sonicated or detergent/sonication-treated spiking material. Furthermore, the filtered sample was ultracentrifuged and subsequently the infectivity was detected in pellet and supernatant fractions after ultracentrifugation. These results showed that 15 nm filtration which is the filter of smallest pore size for virus removal removes infectious prion protein effectively but not completely under the filtration condition of antithrombin preparation. Other prion removal options such as other filter devices, column chromatography and fractionations during processing steps have also been reported [13]. One should choose a suitable spiking material for a process evaluation study, before starting the study. The combination of several different process steps for prion removal is likely to improve the removal of all forms of potential prion contamination and thus safeguard against contamination.

The results of this study also revealed that some infectious prion protein was less than 15 nm in diameter, apparently as a low molecular weight and/or soluble form. Unfortunately, the properties or presence of such a soluble-like infectious prion protein in blood have not been clarified. The properties of this form could be very important to evaluate the risk of prion contamination in biological products. Hence, further investigations are required, especially of the properties of soluble-like prion protein in blood and plasma.

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総　説

バイオ医薬品におけるプリオンの問題 —ヒト赤血球を原料とする人工酸素運搬体をめぐる問題—

Current Situation of Prion Risks on Biological Products - Points on the Consideration for Prion Risk in Artifical Blood Products Derived from Human Red Cells -

柚木 幹弘[1,2,3]，萩原 克郎[2]，生田 和良[3]

Mikihiro Yunoki[1,2,3], Katsuro Hagiwara[2], Kazuyoshi Ikuta[3]

和文抄録

生体成分を原料とするバイオ医薬品やヒト血液成分を原料とする血漿分画製剤には感染性病原体の理論的混入リスクが存在する．そのため，製造工程における安全対策の導入と製造工程中の感染性病原体の不活化・除去能力の評価が求められている．本稿では，ヒト血液由来の赤血球を原料とする人工酸素運搬体を中心に，注目すべき感染性病原体のひとつであるプリオンの問題に関して，1）疫学などの背景，2）輸血領域で現在とられている施策，3）バイオ医薬品のプリオンに対する安全対策研究の現状，などについて概説する．

Abstract

Biological products derived from animal materials and human blood theoretically carry the possible risks of contamination with infectious pathogens. Therefore, to employ the safety measures against the risks during manufacturing process, evaluation for pathogen inactivation and/or removal ability during the manufacturing process steps in individual products are required. Prion issue is an important risk factor of the biological / blood products. In this manuscript, points to consider the above possibility such as 1) epidemiology and background of prion issue, 2) safety measures against prion risks in blood transfusion and blood products fields, and 3) current situation through several researches against prion risks in the field, are summarized.

Keywords

Prion, Biological product, Blood, Safety, vCJD

1．はじめに

抗体医薬を始めとするバイオ医薬品の基本的な製造手法は確立されており，原料や細胞由来の病原体混入リスクや製造工程における病原体等の不活化・除去能力の評価に関する多くの知見が集積されている．血液製剤や血漿分画製剤についても，多くの研究や対策の実施，教訓によって得られた数多くの知見の集積がある．一方，新しい領域の血液代替物は開発途上であるが故に，これらのリスク評価は研究成果の集積途上にあると言える．このような製剤には，その製剤特性を理解した上でバイオ医薬品や血液・血漿分画製剤で得られた知見からそのリスクの全容を俯瞰し，製造工程をデザインした上で，その製剤のリスクを評価する必要がある．本稿では研究開発途上の血液代替物の一つであり，日本の献血血液由来の赤血球を原料とする人工酸素運搬体を中心にバイオ医薬品におけるプリオン問題の現状について概説する．

2．BSE及びvCJDの発生

1986年，英国においてこれまでに確認されていない神経症状を呈するウシの疾患が流行していることが報告された．この疾患は狂牛病（Mad Cow Disease）と一般的に報道されたが，学

(1) ㈱ベネシス安全管理部 品質安全対策グループ　〒541-8505　大阪市中央区北浜2-6-18　Pathogenic Risk Management, Benesis Corp. Pathogenic Risk Management, Benesis Corporation, 2-6-18, Kitahama, Chuo-ku, Osaka, 541-8505 Japan.

(2) 酪農学園大学大学院獣医学研究科 病原体リスク管理学講座　School of Veterinary Medicine, Rakuno Gakuen University

(3) 大阪大学微生物病研究所 ウイルス免疫分野　Department of Virology, Research Institute for Microbial Diseases, Osaka University

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術的には伝染性海綿脳症（Transmissible Spongiform Encephalopathy, TSE）のひとつであり，ウシ海綿状脳症（Bovine Spongiform Encephalopathy, BSE）と名付けられた．このBSE発生の原因が，ヒツジのTSEであるスクレイピーに感染した個体を含む肉骨粉の摂取によることが指摘され，BSEは羊のTSEの原因物質による感染によって生じることが疑われた．英国の公衆衛生当局は，英国内の家畜飼料からこの肉骨粉を排除する決定と，ウシ全頭モニタリング制度を導入することにより，更なる流行の拡大を抑止した．BSEは全世界的に流行する事が懸念されたが，そのほとんどが英国での発生にとどまり1992年をピーク（年間発生頭数約37,000頭）にその発生は減少した（Fig. 1）[1,2]．

日本においては2001年にBSEの発生が初めて確認され，その後，英国と同様の全頭モニタリング制度の導入と全頭検査が導入された．日本では2010年12月現在で総計36例の発生が確認されている[3]．

ところが，1996年に英国においてこれまでに確認されていない病態を呈するクロイツフェルトヤコブ病（Creutzfeldt-Jakob Disease, CJD）が報告された．この新しいヒトの疾患はこれまでのCJDとは違い若年性であることが特徴であるとされ，変異型CJD（Valiant CJD, vCJD）と命名された．このvCJDはBSE同様にほとんどが英国で発見され，その流行パターンがBSEのパターンと相関関係にあることから，vCJDの原因がBSE発症ウシ由来の原料の摂取によるものと疑われた．このvCJDはBSE同様に全世界的に流行・拡大することが懸念されたが大流行には至らず，全世界の現時点での総発生数は221名となっている．一番多く発生した英国では2000年に28例の発生をピークとした現時点の総計174名（内，生存4例），続いてフランスの2005-2006年の6例をピークとする総計25名であった（図1）[2,4]．日本においては2004年にvCJD患者が1名（英国渡航歴有）発生している[5]．

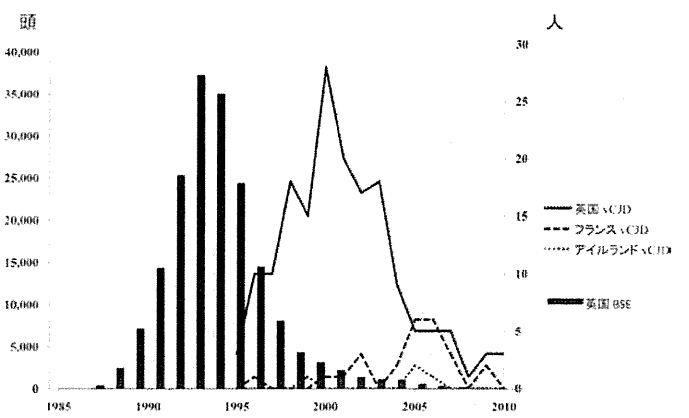

Fig. 1. BSEとvCJDの流行経過（引用1）2）より改変）．

3．伝達性海綿状脳症の原因物質であるプリオン蛋白

CJD/vCJD, BSE, ScrapieなどのTSEの原因物質については長い間論争があったが，1982年にPrusinerがプリオン説を提唱したことで一定の収束を得た[6]．プリオン（Prion）とは蛋白性の感染性粒子という意味であるProteinaceous Infectious Particleの短縮形造語であり，この感染性病原体はウイルス等と異なり遺伝情報を持たない蛋白質そのものである．ヒトの正常型プリオン蛋白（Celler Prion protein, PrP^c）はCD230と同じで，第20番染色体にPRNP遺伝子としてコードされている[7]．病原体としての感染性プリオン蛋白質はウイルス等の外来性物質ではなく，宿主の蛋白分解酵素感受性でαヘリックスリッチな構造をしているPrP^cがβシートリッチな構造をとる，蛋白分解酵素抵抗性（Proteinase resistance prion protein, PrP^{res}）の構造に変換したものである．これを異常型プリオン蛋白と呼ぶが，PrP^{res}の種類により蛋白質分解酵素への抵抗性は同じではない．この構造変換はPrP^{res}を種として，体内のPrP^cが次々にPrP^{res}に変化・結合・集積するものと考えられている．また，感染性プリオン蛋白は単量体でなく重合体として存在していると考えられ，粒子径10nm以下の重合体は感染性がなく，20～25nmの粒子径のものが最も感染性が高いとの報告がある[8]．通常病原体はその存続のために宿主に感染・増殖し，遺伝情報の伝達により子孫を増やすというのが一般的な理解である．しかしながら，このプリオンは存続させるべき遺伝情報を持たないことから，これまでの概念に一石を投じることになった．これらのプリオン蛋白は，高次構造の違いを除いて宿主蛋白そのものであることから，新しい感染性病原体の概念がここに確立することになった．

4．社会の反応

BSEとvCJDの関連性が指摘された事は，畜産を中心とした食品安全上のBSE問題を再び惹起する事となった．さらに，感染性プリオン蛋白は蛋白分解酵素に抵抗性であるだけでなく，121℃のオートクレーブ滅菌にも耐えるなど不活化が困難であることから，医薬品領域においても大きな問題を惹起した．医薬品にはゼラチンカプセルなどウシ由来の原材料が数多く使用されていることから，日欧米の規制当局はプリオンに関するガイダンスの制定や行政指導をおこなった．厚生労働省も，1996年にメーカーに対してウシ由来原材料の使用実態と原産国の把握を求めた[9]，そしてプリオンに対するリスク評価を2000年に求めた[10]．メーカーはこのBSE問題に対応するとともに，可能なものについてはウシ由来以外の原料への切り替えを行った．

ヒトや動物組織を原料とする蛋白医薬製剤は，その製剤化工程で強力な蛋白変性操作は導入されていない．そこで，ヨーロッパの規制当局は1998，2003，2004，2009，2010年にプリオンのリスク評価に関する見解を発出した[11,12,13]．日本においても2003年に厚生労働省よりプリオン対策強化が通知された[14]．これを受け，ウシ組織抽出物由来の医薬品や血液製剤のメーカーは各蛋白医薬製剤に導入されている工程のプリオン除去能力

の評価を行い，必要に応じてプリオンに対するリスク低減のために，ウシ原産国の変更や新たな工程の導入を実施した．

5．CJDの疫学

ヒトのプリオン病であるCJDには孤発性CJD（Sporadic CJD, sCJD），家族（遺伝）性CJD（Familial CJD, fCJD），医原性CJD（iatrogenic CJD, iCJD）そしてvCJDがある．sCJDは55歳以上のヒトに約100万人に1人の割合で発症するCJDであるが，輸血などを通じてのヒトーヒト感染例の報告は現時点ではない．医薬品への感染性プリオン蛋白の混入リスクが高いもののひとつに，輸血を始めとする血液製剤がある．献血後にvCJDに感染していることが判明したドナー由来の血液製剤を輸血された事例が英国で確認され，現時点で受血者4名がvCJDに感染したと考えられている[15]．2010年のWHO見解ではヒト血液は低感染性組織のカテゴリーに分類され，vCJDの感染性あり，CJDは感染性なしと判断されている[16]．更に，英国では血友病患者もモニタリングされている．本年，死亡した血友病患者の病理解剖を行ったところ，脾臓に異常型プリオン蛋白が発見された[17]．この患者は生前に神経症状を発症していなかったにも関わらずvCJDに感染していた．英国健康保健局（Health Protection Agency, HPA）及び米国食品医薬品局（Food and Drug Administration, FDA）は見解を出したが，そのリスクレベルの変更は行わなかった[18,19]．この事例は輸血製剤だけではなく血漿分画製剤でもリスクが残存することを示すことになった．2010年，欧米の規制当局はそれぞれ，メーカーが実施する製剤のリスクアセスメントに関するガイダンス（改定案）を発表した[11,13,20]．また，他の医原性感染として硬膜移植，ヒト脳下垂体由来製剤（成長ホルモンなど），角膜移植や手術器具を介しての感染も認められている[21,22]．

日本ではCJDのサーベイランスが実施されており，1999年から2009年までの間に確認された1324例のCJDは，sCJDが1019例（77％），fCJDが220例（16.6％），iCJD（全例硬膜移植）が80例（6.0％，期間外を加えると総計138名）），vCJDが1例（0.1％），分類できないCJDが4例（0.3％）であった[22]．この中で実際に輸血によるリスクがあると考えられるドナーとなりうるのはiCJDとvCJD患者由来であるが，手術歴などの確認によりiCJD患者がドナーになる可能性を排除しており，現実にはvCJDのリスクを検討する必要がある．日本のvCJDは現時点で1例であるが，英国渡航に由来してvCJDに感染するリスクと，その感染者が献血し，輸血を通して国内で感染が拡大するリスクを検討したところ，2007年までに0.06人が発症し，ポアソン分布を適用すると少なくとも1人の発生は否定できないが，2人以上の発生は極めて低いという結果が得られた．次に輸血による感染予測が行われた．モデル予測は，1）1990年に20代前半の感染者が1名存在し，2）輸血感染による潜伏期間は食餌による感染時と同じ，3）汚染血の輸血で必ず感染，4）輸血歴のある人や海外渡航歴のある人の献血制限は実施しない，5）輸血経験者の死亡率は増加しないという条件で実施した．その結果，感染者数は2003年ごろにピークを迎え，累積発症者数の増加は2010年代の前半でほぼ終わり，0.563人という結果となった．これらの結果から，現在のBSE/vCJDの疫学状況等の知見が大きく変化しない限り，2010年以降の日本における輸血によるvCJD感染が現実に発生する可能性は非常に低いと考えられる[23]．

6．血液及び血液成分のリスクとその低減策

プリオンリスクの実効的対策は，検査によるリスク排除である．現在，日本ではウシに対して世界でも例を見ない全数検査を行っており，世界的にも最高レベルの対策を取っている．これに加えて，餌の管理，臨床症状のモニタリング，イヤータグによる全数管理，屠場での解体方法や交差汚染の管理など，多くの対策を組み合わせることによってその安全性を確保している．ヒトのvCJDについてもウシの場合と同様に多数の検体を迅速に安価に検査する方法を開発する必要がある．vCJD検査の産業応用できる手法として，蛋白分解酵素を用いないELISA法が提案されている[24]．しかしながら産業応用可能でCJD/vCJDに有効な検査手法は現時点で開発されていない[25]．

血液は主に赤血球，血小板，白血球，血漿の画分に分けることができる．プリオン蛋白は細胞膜上に存在すると考えられていることから，血液細胞が輸血領域における主なプリオン伝播リスクであると考えられる．輸血によるvCJD感染リスクの低減策を講じる必要があるが，献血基準の中にCJD/vCJDリスクを低減させる問診項目として英国などの滞在歴を設定し，ドナーの適格性を確保している．また，白血球除去フィルターがプリオン除去に効果があるとされ，その効果が確認されているものもある[25,26]．血漿分画製剤の原料となる血漿については，更に6ヵ月の貯留保管を行った後に日本赤十字社からメーカーに出荷されることにより，採血（原料）段階でのリスク低減を行っている．更に，血漿分画製剤の製造工程においてプリオンを除去する工程を導入することによりその安全性の確保に努めている[27]．

7．人工酸素運搬体

現在開発研究が進められている人工酸素運搬体の種類はいくつかあるが，その多くはヒト赤血球よりヘモグロビンを抽出・精製した高純度・高濃度のヒトヘモグロビン（Hb）溶液を原料とし，脂質二重膜で包埋したリポソーム型又は非カプセル型架橋修飾・PEG修飾の形状をとる[28]．ヒト赤血球の大きさは約8μm，精製したHbは約4 nmの大きさであり，これを小胞体に包埋した時の小胞体径は約200～250nmである[28,29]．これらの運搬体はHbをヒト赤血球から得ていることから，ドナー由来の病原体の混入が理論的なリスク因子として存在する．この人工酸素運搬体に使用されるHbは赤血球を出発原料として精製されるが，Hbの大きさが約4 nmに対して，異常型プリオン蛋白単分子の分子量は約30,000，感染性プリオン蛋白の粒子は10nm以上の重合体[8]と考えられる．Hbの精製工程におけるプリオン除去は血漿分画製剤の製造工程に導入されているプリオン除去手法を応用可能であると考えられ，人工酸素運搬体の製

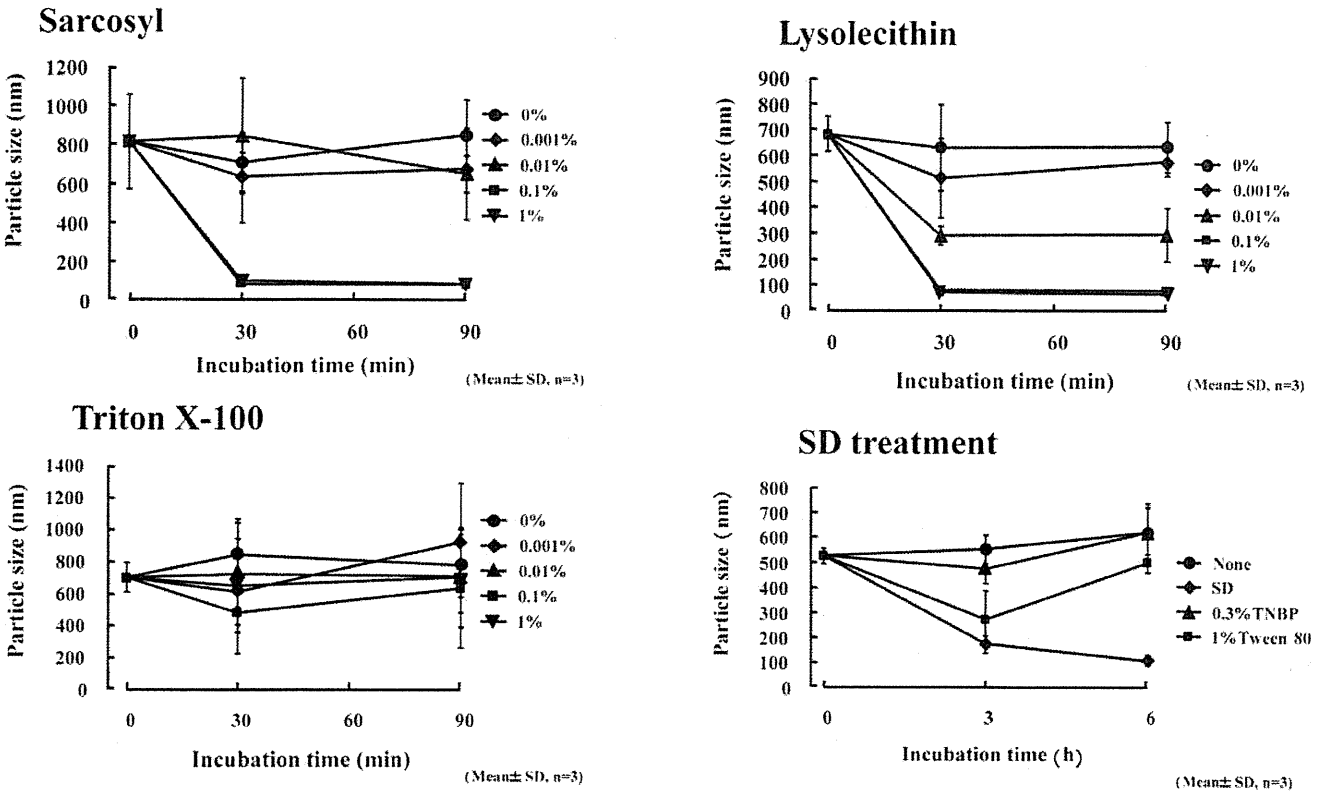

Fig. 2. 界面活性剤処理による263K株感染ハムスター脳由来マイクロソーマル画分の粒子径変化（文献30より引用）.

造工程にプリオン除去工程を導入するには赤血球からヘモグロビンを精製する過程に導入するのが適している.

8．工程評価を行う上でのプリオン材料調製法の問題

プリオン除去に関する工程評価を行う場合，感染動物の脳を出発材料として調製することが一般的である．プリオン研究初期には脳ホモジネート（Brain Homogenate, BH）を用いていたが，その後は精製操作を加えて膜画分（Microsomal Fraction, MF）が主に使われるようになった．本来，工程評価に用いるプリオン材料は感染動物の血液や血漿を用いることが望ましい．しかしながら，血液から工程評価に用いることができる量の感染性プリオン蛋白を得ることは困難であり，このMFを使用せざるを得ない．しかしながらMFは工程評価に用いる材料として最適化されたものではなかった.

そこで私たちは，ヒツジのスクレイピー株（Hamster adopted scraipieハムスター263K）を用いてこのMFの粒子径に着目した工程評価材料としての適格性を検討した．MFの平均粒子径は約800nm程度あったのに対して，Sarcosyl やLysolecithin や超音波処理によって短時間で，約200nm以下の平均粒子径にすることができた．ウイルス不活化に用いられるSD処理は用いられるTri (n-butyl) phosphate（TNBP）とPolyoxyethylensorbitan monooleate（Tween80）を組み合わせて長時間処理しないと平均粒子径を200nm以下に出来なかった．これに対して，Polyoxyethylene-p-isooctylphenol（Triton X-100）は平均粒子径に影響を与えなかった（Fig. 2, 3）[30]．また，MFをPBSに添加し220nm，100nm，75nm，35nm，19nm，15nmのろ過膜でろ過したところ，100nmと75nmのろ過膜の結果において超音波処理の有無で除去効果（除去指数，Log Reduction Factor, LRF）に差が生じた（Table 1）[30]．このことより，工程評価に用いるプリオン材料はSarcosilやLysolecithinなどの界面活性剤処理や超音波処理を行う事が望ましいことが明らかになった．なお，超音波処理

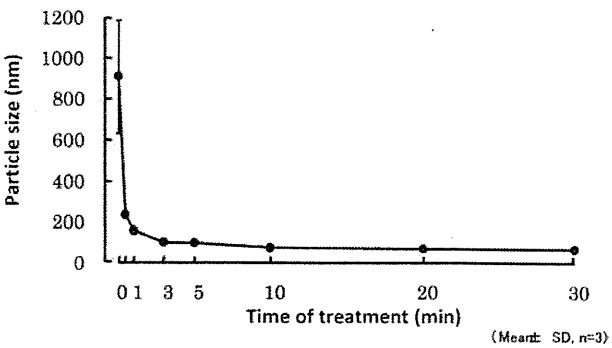

Fig. 3. 超音波処理による263K株感染ハムスター脳由来マイクロソーマル画分の粒子径変化（文献30より引用）.

Table 1. PBS中の263K株異常型プリオン除去（文献30より引用）.

	PVDFフィルター				Planovaフィルター					
公称膜孔径	220nm		100nm		72nm		35nm		15nm	
超音波処理	あり	なし	あり	なし	あり	なし	あり	なし	あり	なし
ろ過前	4.2 / 3.5	3.5 / 4.2	4.2 / 3.5	3.5 / 4.2	4.2 / 4.2	3.5 / 4.2	4.2 / 4.2	3.5 / 4.2	4.2 / 4.2	3.5 / 4.2
ろ過後	3.8 / 3.8	3.1 / 3.8	3.8 / 3.1	2.4 / 3.1	2.4 / 2.4	<1.0 / <1.0	<1.0 / <1.0	<1.0 / <1.0	<1.0 / <1.0	<1.0 / <1.0
除去指数	0.4 / -0.3	0.4 / 0.4	0.4 / 0.4	1.1 / 1.1	1.8 / 1.8	≥2.5 / ≥3.2	≥3.2 / ≥3.2	≥2.5 / ≥3.2	≥3.2 / ≥3.2	≥2.5 / ≥3.2

ウエスタンブロッティング（WB法）による評価.
数値はNon-detectable endpoint法によって求めた総Log_{10}値で示した.

したMFは組成の変化がなく，工程サンプルに与える影響は最小限に止めることができるが，界面活性剤処理したMFは界面活性剤が残存することから工程サンプル及び評価に与える影響を考慮する必要がある.

次に，このプリオン材料が工程評価を行う上で，血液中の感染性プリオン蛋白をどこまで反映・再現させているかという問題がある．輸血でvCJDが伝播することが報告されており，血液細胞画分には膜結合蛋白であるプリオン蛋白が含まれることは予測できる．しかしながら血漿には感染性プリオン蛋白が細胞断片として含まれるのか，それとも可溶化画分として存在するのかについては不明な点が多い．私たちは血漿分画製剤の製造工程に導入されているウイルス除去膜（平均膜孔径15nm）を用いて，実際の製造条件を模倣してプリオン蛋白の除去性能を評価した．この15nmのろ過膜はバイオ医薬品分野に産業応用できる世界で最も公称膜孔径の小さなウイルス除去膜である．この膜処理により，ウエスタンブロッティング（WB）法による評価でろ液中の異常型プリオン蛋白は検出限界以下にまで除去され3.5Log以上の除去指数を示した．これに対して，動物への接種実験ではろ液中の感染性プリオン蛋白は4.0Log以上の除去指数を示したものの，一部のサンプルで感染性を認めた．更にこの15nmろ液を150,000g，1時間の超遠心操作を行い，得られた上清画分と沈殿画分について感染実験を行った．この超遠心条件はプリオン蛋白を濃縮する時に採用されている条件であるが，驚くべきことに両方の画分に感染性が認められた（Table 2）[30,31]．感染動物の脳ホモジネートの超遠心上清に感染性が認められることはBerardiらが報告している[32]が，この上清画分に残存する感染性プリオン蛋白が15nmの膜を通過することが可能であることは新しい知見である．一方，Silveiraらは約20～25nmの重合体が最も感染性が高く，10nm以下の画分には感染性は認められなかったと報告している[8]．15nmのウイルス除去膜はその孔径に近い粒子径を有するパルボウイルスなどを高負荷した時に一部のウイルスがろ液に認められることを経験している[33]が，15nm以下の大きさの感染性プリオン蛋白が膜を通過した可能性がある．一方，vCJD感染マウスの，赤血球，Buffy coat，血小板含有血漿，血漿等を正常マウスに

Table 2. 15nmろ過による異常型プリオン蛋白の除去（文献30, 31より引用）.

	Lysolecthin及び超音波処理263K		超音波処理263K	
	WB（定量）	BA（定量）	WB（定量）	BA（定性）
ろ過前	6.1 / 6.1	7.97 / 8.30	3.6	発症
ろ過後	<2.6 / <2.6	<3.25 / 4.30	<0.8	発症
除去係数	≥3.5 / 3.5	≥4.72 / 4.00	≥2.8	NA
超遠心沈殿	NA	NA	<1.0	発症
超遠心上清	NA	NA	<1.0	発症

WB：ウエスタンブロッティングによる評価.
数値はNon-detectable endpoint法によって求めた総Log_{10}値で示した.
BA：動物接種試験による定量又は定性評価.
定量評価は階段希釈サンプルを用い一群6匹の結果からKarbar法により，総ID50（Log_{10}）値で示した.

脳内又は静注にて接種したところ，赤血球画分には感染性が認められなかったものの，それ以外のサンプルすべてに感染性が認められた[34]．これらの結果はあくまで実験的に得られた現象であり，ヒト血漿中の存在様式を証明したものではないが，感染性プリオン蛋白はこれまでに知られている膜結合型としての存在様式に加えて，血漿中に可溶化又は非常に微細な画分（私たちは，Soluble like formとした）としても存在し得ることを示唆することとなった.

赤血球を原料とするHbを考える場合，混入してくるプリオン蛋白の形態として，膜由来（結合）とSoluble like formのプリオン蛋白の2つの存在様式が考えられる．血漿も同様に細胞断片及びSoluble like formの存在様式が理論的に考えられる．現在評価試験に用いているプリオン材料はその両方を含んでいることが実験結果から示されているので，評価結果は両方の存在様式を反映したものと思われる．しかしながら，Soluble like formの感染性プリオン蛋白の実態や血漿中の感染性プリオン蛋白の存在様式に関する知見はほとんどないので，Soluble like formの意味についての更なる研究が求められる.