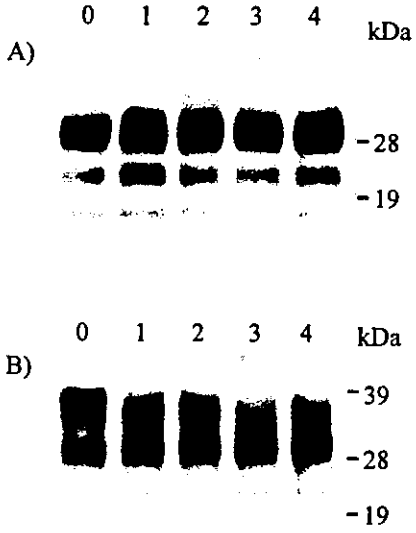

Fig. 3. Western blotting analysis of PrP^{Sc} from tissue incubated at 37°C in Exp. 2. Numbers at the top of each lane indicate days of incubation. PrP signals were detected with mAb T2. (A) Samples treated with PK. (B) Samples prepared without PK treatment. Despite the remarkably low OD value on the ELISA, PrP^{Sc} in the 2-day incubated tissue represented in lane 2 showed no significant loss and no more severe partial digestion than the other samples. The equivalent of 500 μg of wet brain tissue was loaded in each lane. Molecular mass markers (kDa) are shown on the right.

peat sequence) (Figs. 3A and B, lanes 2). Therefore, essentially no differences in WB results were observed between Exp. 1 and 2 (Figs. 2 and 3).

Inhibition of ELISA: The results of WB suggested that the markedly low OD value of the 2-day-incubated sample in Exp. 2 was not caused only by digestion of the N-terminal sub-region of PrP^{Sc} during incubation of the brain tissue. It was also noted that the OD values of the samples diluted 1:4 tended to be higher than those of undiluted samples (Fig. 1B). These findings led us to hypothesize that the 2-day-incubated sample with severe deterioration contained some inhibitor(s) for reactivity in the ELISA. Therefore, we investigated the possible presence of an inhibitor in the deteriorated brain samples. Samples were prepared for the ELISA from fresh or artificially deteriorated BSE-negative (normal) cattle brain tissue, and then mixed with an equal volume of a sample prepared from fresh BSE-positive cattle brain tissue. The ELISA procedure was carried out following the manufacturer's instructions. As shown in Table 1, in the sample incorporating normal brain tissue incubated for 2 days at 37°C, detection of PrP^{Sc} by the ELISA was clearly inhibited. For the control sample, containing fresh normal cattle brain tissue, no inhibition of the ELISA was observed, relative to buffer.

Table 1. Inhibition of ELISA by deteriorated normal brain samples

Incubation time of normal samples (days)[a]	Heating time in preparation (min)[b]	OD value (450/620 nm)[c]
0	5	3.09 ± 0.13
	30	2.59 ± 0.11
2	5	0.22 ± 0.00
	30	0.97 ± 0.06
Buffer		2.06 ± 0.06

a) Normal brain tissue was incubated at 37°C for 2 days to induce deterioration (2 days), and then mixed with equal volumes of fresh BSE-positive brain samples. Normal brain without deterioration (0 days) was used as the control.
b) Before mixing with the BSE-positive sample, the normal brain samples were boiled for 5 or 30 min.
c) Average ± standard deviation.

In the manufacturer's protocol, the solubilization step for the preparation of ELISA samples includes a boiling time of 5 min. When the boiling time was prolonged to 30 min, some recovery of ELISA reactivity, relative to inhibited OD values, was noted (Table 1).

DISCUSSION

In the present evaluation, PrP^{Sc} could be detected with the Platelia BSE kit in all artificially deteriorated samples incubated for up to 4 days, but a reduction in the OD values was observed in the samples incubated for 2 days or more at 30°C, or for 1 day or more at 37°C. It has been reported that, like PK, several other proteases digest PrP^{Sc}, cleaving it to PrPcore [9, 16]. As the BSE-positive brain tissues used in this study were not aseptic, it is likely that PrP^{Sc} was partially digested by contaminating bacterial proteases in addition to endogenous enzyme(s) and thus lost its N-terminal region during the incubation. The capture antibody used in the Platelia BSE kit recognizes the octarepeat sequence (personal communication with Mr. K. Sugimura from Nippon Bio-Rad Laboratories, Tokyo, Japan), which is adjacent to the N-terminal of PrPcore and therefore sensitive to protease digestion so that loss of the antibody-recognition sequence may cause the reduction in the OD value in the ELISA. This was strongly supported by the WB results that we obtained when PK treatment was omitted from sample preparation (Figs. 2B and C, and 3B).

There was a discrepancy between the ELISA results and those for WB. Although the OD values were significantly decreased after 2 days at 30°C or 1 day at 37°C, prolongation of incubation thereafter did not cause any further decrease, with the aforementioned exception of the 2-day-incubated sample in Exp. 2. On the other hand, WB showed a gradual reduction in the 32–36-kDa band and a reciprocal increase in the 27–30 kDa band depending on the incubation time in both experiments. This discrepancy may have been partly owing to the different antibodies that were used for detection of PrP^{Sc} in WB and in the ELISA, but the reason is

not clear.

WB revealed that PrP^{Sc} was not always completely digested to PrPcore, even after 4 days of incubation (Figs. 2B and C, and 3B). In this study, we used brain tissues from BSE-affected cattle with typical clinical symptoms. Enough PrP^{Sc} was evidently contained in the original tissues to compensate, in the ELISA, for the loss of the N-terminal moiety of PrP^{Sc} by deterioration. In the pre-clinical or early stages of infection, the amount of PrP^{Sc} in brain tissue might be far less. In such cases, it is probable that deterioration would seriously compromise the ELISA results.

The sample incubated for 2 days in Exp. 2 had an especially low OD value (Fig. 1B), but the PrP band pattern of the sample in WB (Fig. 3B, lane 2) was similar to that of the 2-day-incubated sample in Exp. 1 (Fig. 2B, lane 2). It is difficult to entirely explain this low OD value by partial digestion of PrP^{Sc} accompanying the deterioration. Thus, participation of an inhibitor(s), generated by the deterioration of brain tissues, was suspected, as suggested by the data in Table 1. The observed increase in the OD value of the 2-day-incubated sample diluted 1:4, relative to the undiluted sample, suggests that the effect of the inhibitor(s) is decreased by dilution. The inhibitor(s) also seems to be partially thermolabile, since prolonged boiling decreased inhibition in the deteriorated sample, as seen in Table 1. Therefore, the OD value of the ELISA in deteriorated samples seems to be affected not only by partial digestion of PrP^{Sc}, but also by the presence of some inhibitors of ELISA reaction.

In this study, we pooled brain tissues from several BSE-positive cattle, and thoroughly minced and mixed them before use. In Exp. 2, minced tissues were aliquoted into 5 separate tubes prior to incubation. As the original tissues were not aseptic, and as the minced and mixed tissues were not completely homogeneous microbiologically, due to the presence of some small lumps, massive deterioration might have occurred because of the proliferation of contaminating bacteria in the tube of the 2-day-incubated sample. Although it is almost impossible to verify the degree of deterioration of brain tissue within the skull of a cattle carcass, our results seemed to indicate the need to be cautious regarding the possible presence of ELISA inhibitors in severely deteriorated tissue. To avoid or minimize the generation of such inhibitors, tissue specimens must be collected as soon as possible when fallen cattle are found.

Another problem in testing fallen stock for BSE may arise from unequal distribution of PrP^{Sc} in BSE-affected brains. Spongiform changes and accumulation of PrP^{Sc} are most frequently observed in the obex region [15, 18], but, it could be quite difficult to collect the obex region precisely from extensively deteriorated and liquefied brain tissue. Furthermore, in such cases it would be difficult to perform IHC as a confirmation test.

It has been shown that sample autolysis does not affect detection of PrP^{Sc} by means of WB [3, 5, 13]. Our WB results also demonstrated no reduction in the PrP^{Sc} signal as a result of deterioration at 30°C or 37°C for up to 4 days, as so far examined (Figs. 2A and 3A). In this study, we showed that several problems undermine the utility of the ELISA with deteriorated samples, whereas WB remains very dependable. Therefore, WB might be the only reliable procedure to detect PrP^{Sc} in severely damaged samples from fallen stock.

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Non-glycosylphosphatidylinositol (GPI)-anchored recombinant prion protein with dominant-negative mutation inhibits PrP^{Sc} replication *in vitro*

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Key Words: recombinant prion protein (rPrP), dominant negatives, Q218K, quinacrine, glycosylphosphatidylinositol (GPI)-anchor, lipid rafts, Creutzfeldt-Jakob disease (CJD)

Abbreviations: PrP = prion protein, GPI = glycosylphosphatidylinositol, CJD = Creutzfeldt-Jakob disease, rPrP = recombinant prion protein, EC_{50} = 50% effective concentration, EC_{99} = 99% effective concentration, PrP^C = host-encoded cellular prion protein, PrP^{Sc} = abnormal protease-resistant pathogenic prion protein, TSE = transmissible spongiform encephalopathy, BSE = bovine spongiform encephalopathy, IPTG = Isopropyl-β-D-thiogalactopyranoside, β-ME = β-mercaptoethanol, PMSF = Phenylmethylsulfonyl fluoride, PBS = phosphate buffer saline, PK = proteinase K, WST-8 = 2- (2-methoxy-4-nitrophenyl)- 3- (4-nitrophenyl)- 5- (2,4-disulfophenyl)- 2H- tetrazolium, monosodium salt, SPR = surface plasmon resonance, PIPLC = phosphatidyl-inositol specific phospholipase C

Abstract

Dominant-negative mouse prion protein (PrP) with a lysine mutation at codon 218 (Q218K) is known to inhibit prion replication. In order to gain further mechanistic insight into such dominant negative inhibition, non-glycosylphosphatidylinositol (GPI)-anchored recombinant PrP with Q218K (rPrP-Q218K) was investigated. When applied into scrapie-infected mouse neuroblastoma (ScN2a) cells, rPrP-Q218K but not wild-type rPrP (rPrP-WT) exclusively inhibited abnormal protease-resistant pathogenic isoform (PrP^{Sc}) replication without reducing the viability of the cells. It was even more efficient than quinacrine, which has already been prescribed for sporadic Creutzfeldt-Jakob disease (CJD) patients; 50% effective concentration (EC_{50}) = 0.20 μM, 99% effective concentration (EC_{99}) = 0.86 μM vs. EC_{50} = 0.45 μM, EC_{99} = 1.5 μM. Besides, no apparent cell damage was observed at the concentration of up to 4.3 μM (100 μg/ml). In combination treatment with 0.43 μM (10 μg/ml) of rPrP-Q218K, EC_{99} of quinacrine was decreased from 1.5 μM to 0.5 μM, and the cell viability was recovered from 50% to over 90% as inversely proportional to the concentration of quinacrine. Such combination could alleviate the side effects of quinacrine by reducing its effective concentration without changing or even acceleration the inhibition efficacy. Since homogeneous, high-quality rPrPs could be easily prepared from Escherichia coli in large quantities, rPrP-Q218K is a good candidate for a prion replication antagonist.

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Introduction

Human prion disease or transmissible spongiform encephalopathy (TSE), such as sporadic Creutzfeldt-Jakob disease (CJD) and variant CJD transmitted from bovine spongiform encephalopathy (BSE) constitutes a group of invariably fatal neurodegenerative disorders[1,2]. Prion protein (PrP) consists of two isoforms, one is a host-encoded cellular isoform (PrP^{C}) and the other is an abnormal protease-resistant pathogenic isoform (PrP^{Sc}). The latter is a causative agent of prion disease. PrP^{Sc} stimulates the conversion of PrP^{C} into nascent PrP^{Sc}, and the accumulation of PrP^{Sc} leads to the central nervous system (CNS) dysfunction and neuronal degeneration[3].

A human polymorphic lysine variant at codon 219 (E219K) in the Japanese population, known to render humans resistant to sporadic CJD[4,5], acts as a dominant negative in scrapie-infected mouse neuroblastoma (ScN2a) culture cells after gene transfection[6,7] and transgenic mice expressing lysine at codon 218 in mouse PrP (mouse Q218K, which corresponds to human E219K)[8]. Of note, such a genetic population with E219K and the transgenic mice with Q218K complete their life span with no apparent phenotypic abnormality[5,8].

We now demonstrate that administration of non-glycosylphosphatidylinositol (GPI)-anchored recombinant PrP (rPrP) with Q218K mutation (rPrP-Q218K) but not wild-type rPrP (rPrP-WT) exclusively inhibited the PrP^{Sc} formation in ScN2a cells, even more efficiently than quinacrine, which has already been prescribed for CJD patients, and no apparent cell damage was observed up to 5-fold higher concentrations of a 99% effective concentration (EC_{99}). When combined, rPrP-Q218K efficiently reduced the effective dosage of quinacrine, and thus rendered ScN2a culture cells more viable. Such a combination could alleviate the side effects of quinacrine by reducing its effective concentration without changing or even accelerating the inhibition efficacy. Since homogeneous, high-quality rPrP could be easily prepared from *Escherichia coli* in large quantities, rPrP-Q218K might be a good candidate as a prion replication antagonist.

Materials and methods

Expression plasmid construction

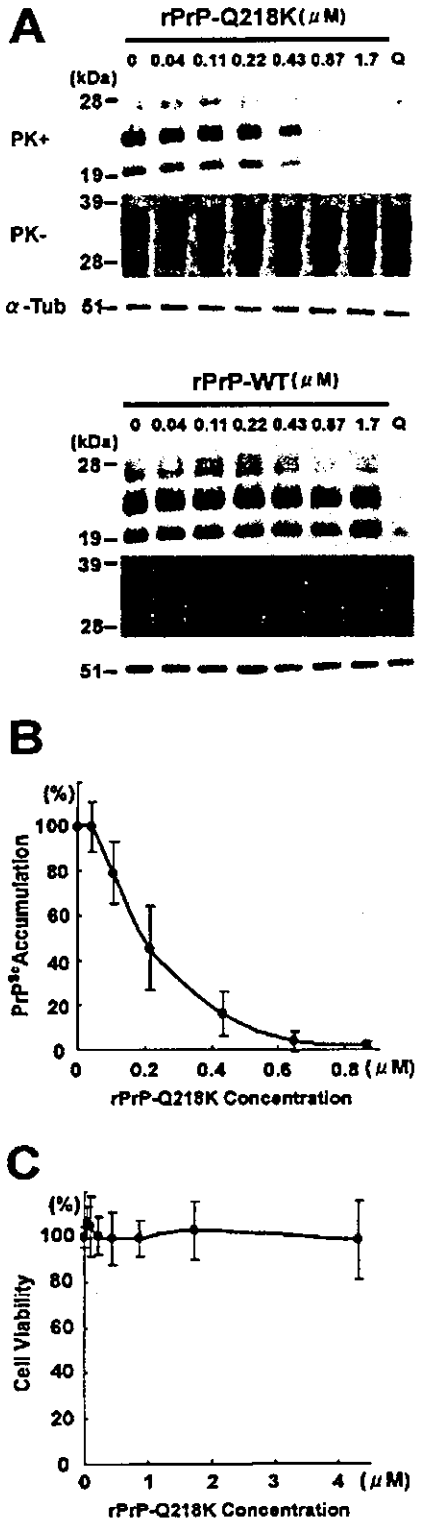
The gene, mouse (Mo) PrP(23-230), coding for residues 23-230 of mouse PrP was PCR-amplified from mouse brain cDNA using the oligonucleotide primers (5′-GGAATTCACCATGAAAAAGCGGCCAAAGCCTGGAGGG-3′ and 5′-CCGCTCGAGTCAGGATCTTCTCCCGTCGTAATAGGC-3′) and cloned via *EcoRI* and *XhoI* sites into the plasmid pBluescript II SK(+) (Stratagene, La Jolla, CA). The genes for 3F4-tagged MoPrP (MHM2PrP) were also cloned using PCR amplification from pSPOX-MHM2PrP[9,10] as above. The Q218K mutation was generated by PCR-directed mutagenesis using primers (5′-ATGTGCGTCACCCAGTACAAAAAGGAGTCC-3′ and 5′-ATAGGCCTGGGACTCCTTTTTGTACTGGGT-3′). The DNA fragments were cloned into a modified pET-11a (Invitrogen, Carlsbad, CA), pEY2, of which *EcoRI* and *XhoI* sites were introduced as multicloning sites, via *EcoRI* and *XhoI* sites.

Purification of recombinant prion proteins (rPrPs)

The rPrPs were expressed as inclusion bodies in the *E. coli* BL21(DE3) (Stratagene) in the presence of 0.1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). The inclusion bodies were collected from sonicated lysates by centrifugation at 27,000 × g for 10 min, washed three times in Buffer A (2 M urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM β-mercaptoethanol (β-ME), 0.5 mM Phenylmethylsulfonyl fluoride (PMSF)), and solubilized in Buffer B (8 M urea, 25 mM Tris-HCl, pH 7.5, 2 mM β-ME, 0.5 mM PMSF). After centrifugation (200,000 × g, 30 min), the supernatant was applied to a CM-Sepharose column (Amersham Bioscience, Piscataway, NJ), washed with Buffer B containing 100 mM NaCl and eluted with Buffer B containing 150 mM NaCl. The eluate containing rPrP was applied to an Ni-NTA agarose column (Qiagen, Valencia, CA), washed with Buffer B containing 5 mM imidazole and eluted with Buffer B containing 200 mM imidazole. The eluate was diluted 10-fold 1 M arginine-HCl, pH 8.0, 1 mM reduced glutathione, 0.8 mM oxidized glutathione and incubated at 4°C overnight. After incubation at 37°C for 10 min, the refolded recombinant proteins were concentrated and buffer-changed into phosphate buffer saline (PBS) by Ultrafree-15 10K NMWL (Millipore, Billerica, MA). Concentrations of rPrP were calculated by the absorbance at 280 nm with specific absorbance unit ($A_{280nm,\ 1mg/ml,\ 1cm}$) of 2.70.

Inhibition assay of PrP^{Sc} accumulation in ScN2a cells

ScN2a cells were grown and maintained as described[11]. Twenty-four hours after splitting, cells were incubated in a fresh medium containing the appropriate concentration of rPrP and/or quinacrine (Sigma, St. Louis, MO) or the same volume of PBS as a negative control and incubated for 3 days. Quinacrine was dissolved in PBS. Cell lysis and proteinase K (PK) digestion were performed as described[12]. PK-insoluble pellets and PK-undigested samples were subjected to 12% SDS-PAGE and Western blotting using standard procedure. Anti-PrP



monoclonal antibody (mAb) 6H4 (1:5000; Prionics, Schlieren, Switzerland) or α-tubulin mAb (1:10000; DM1A, Sigma) was used as the primary antibody, and horseradish peroxydase-conjugated anti-mouse IgG (1:5000; Cappel, West Chester, PA) was used as the secondary antibody. Immunodecorated bands were visualized by the ECL-plus (Amersham Bioscience). For evaluating the accumulation of PrP^{Sc}, the PK-resistant bands were quantified by densitometry (LAS-1000; Fujifilm, Tokyo, Japan). Average values of at least three independent experiments were plotted as percentage of the amount of PrP^{Sc} found in equivalent untreated ScN2a cells on the day of collection.

Cytotoxicity assays

The cytotoxicity of rPrP and quinacrine in ScN2a cells was evaluated by the WST-8 assay (Cell Counting Kit-8, Dojindo Lab, Kumamoto, Japan) measuring the formation of a yellow color formazan dye produced by dehydrogenase activities in viable cells from 2- (2-methoxy-4-nitrophenyl)- 3- (4-nitrophenyl)- 5- (2,4-disulfophenyl)- 2H- tetrazolium, monosodium salt (WST-8). ScN2a cells (4×10^{3} cells/well) were cultured at 37°C for 24 h in 96-well plates, incubated in the medium containing the appropriate concentration of drugs for 48 h and were subjected to WST-8 assay according to the manufacturer's protocol.

FIGURE 1: Dose-dependent inhibition of PrP^{Sc} formation in ScN2a cells with rPrP-Q218K. (A) PrP^{Sc} signals in ScN2a culture cells are compared by immunoblotting in the presence of rPrP-Q218K or rPrP-WT at 0 – 1.7 μM (0 - 40 μg/ml). (0) represents untreated cells, and (Q) represents positive controls treated with 1.5 μM of quinacrine. PrP^{Sc} is detected with anti-PrP mAb (6H4) after proteinase K (PK) digestion (20 μg/ml, 1 h, 37°C, 1st row), and total PrP (PrP^{C} and PrP^{Sc}) is detected without PK digestion (2nd row). After incubation with rPrP-Q218K, PrP^{Sc} in ScN2a cells is reduced in a dose-dependent manner, whereas the administration of rPrP-WT by up to 1.7 μM (40 μg/ml) does not change PrP^{Sc} formation. Total PrP remains unchanged in both treatments. The same undigested cell lysates are stained with αtubulin mAb (DM1A, Sigma, St. Louis, MO). (B) Densitometric measurements of PrP^{Sc} signals in panel A. All data represent the mean values (± SD) from at least three independent experiments. (C) Cell viability is determined by the WST-8 assay, in which the absorbance values indicate the yield of colored formazan in proportion to total number of viable cells. Each point represents the mean absorbance value (± SD) calculated from four sets of experimental data. Up to 4.3 μM (100 μg/ml) of rPrP-Q218K doesn't reduce the viability of ScN2a cells.

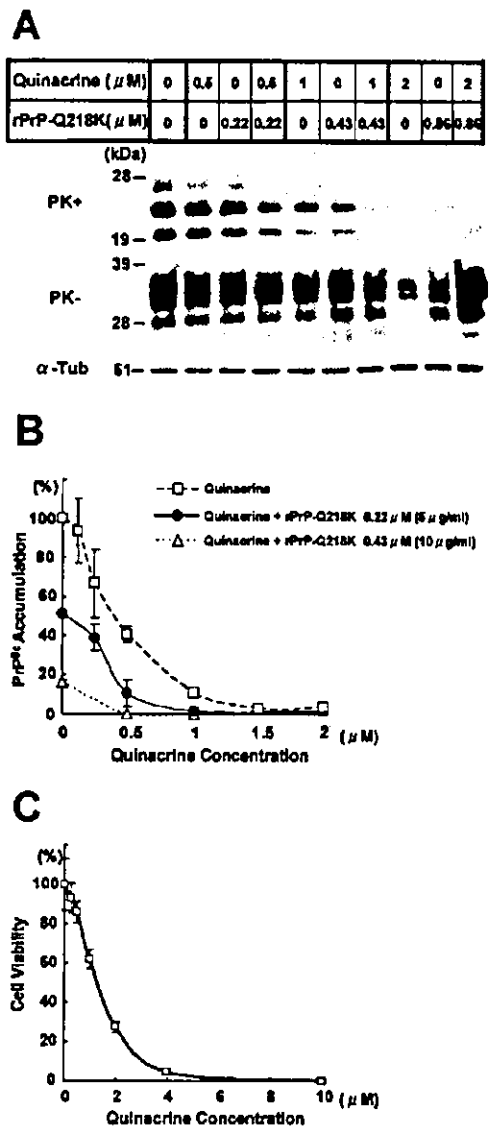


FIGURE 2: Additive inhibition of PrP^{Sc} formation by the combination of rPrP-Q218K and quinacrine. (A) PrP^{Sc} signals in ScN2a cells are compared by immunoblotting in the presence of quinacrine and/or rPrP-Q218K (see legend to Figure. 1A). PrP^{Sc} level in ScN2a cells is additively reduced. (B) Densitometric measurements of PrP^{Sc} signals in panel A (see legend to Figure. 1B). Open squares, quinacrine; filled circles, quinacrine with 0.22 μM (5 μg/ml) of rPrP-Q218K; open triangles, quinacrine with 0.43 μM (10 μg/ml) of rPrP-Q218K. The inhibition on PrP^{Sc} formation with quinacrine with rPrP-Q218K is more effective than that of quinacrine alone. (C) Cell viability is determined by the WST-8 assay (see legend to Figure. 1C). The treatment of quinacrine damages the cell viability in a dose-dependent manner.

Immunofluorescent microscopy

For indirect immunofluorescence analysis, mouse neuroblastoma (N2a) cells grown on glass cover slips in the presence of 0.43 μM (10 μg/ml) of rPrP with 3F4 epitope (MHM2 rPrP) for 3 h were rinsed with PBS without Ca^{2+} and Mg^{2+} (PBS(−)) and then fixed with 2% formalin in 70% PBS(−) for 15 min at room temperature. After four washes, the fixed cells were incubated 10% FBS in PBS(−) for 30 min at room temperature. They were then incubated for 1 h at room temperature with anti-SHaPrP 3F4 mAb (1:200; Sigma) and anti-asialo-GM1 antibody (1:200, CALBIOCHEM, La Jolla, CA) as a marker of rafts. After four washes with PBS (−), the cells were incubated with Alexa 488 Fluor-conjugated goat anti-mouse IgG (1:500, Molecular Probes, Eugene, OR) and Alexa 594 Fluor-conjugated goat anti-rabbit IgG (1:100, Molecular Probes) for 1 h at room temperature. The cells were washed 4 times with PBS(−) and mounted with SLOW FADE (Molecular Probes) and observed using Delta Vision Microscope Systems (Applied Precision, LLC).

Surface plasmon resonance (SPR) measurement

A BIAcore 3000 system (BIAcore AB, Uppsala, Sweden) was used to analyze molecular interactions by means of SPR. rPrP-WT at 500 μg/ml was diluted 1:10 with 10 mM sodium acetate buffer at pH 6.0 and immobilized to a sensor chip CM5 (carboxymethylated dextran surface) using amine coupling according to the manufacture's instructions. Samples for analyte proteins were diluted ($3.2 \times 10^{2\sim0}$ μg/ml) in the running buffer (10 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20), and injected over the surface at 4°C with a flow rate of 20 μl/min. Each sensorgram was subtracted for the response observed in the control flow cell containing a blank surface and results were analyzed by using BIA evaluation SPR kinetic software (BIAcore).

Results

Purified rPrP-Q218K or rPrP-WT was added into the culture media of ScN2a cells at the designated concentrations and incubated for 3 days (see Materials and methods). Ultracentrifugation using a sucrose density gradient revealed that these rPrPs were monomeric (data not shown). While PrP^{Sc} formation was not altered by up to 1.7 μM (40 μg/ml) of rPrP-WT (Figure 1A), it was dramatically reduced in rPrP-Q218K-treated ScN2a cells; 50% effective concentration (EC_{50}) was 0.19 μM (4.5 μg/ml) and EC_{99} was 0.86 μM (20 μg/ml) (Figure 1B) in a concentration-dependent manner (Figures 1A, 1B). Of

note, the viability of ScN2a cells measured with WST-8 assay was not reduced up to 5-fold higher concentrations than EC_{99} of rPrP-Q218K (Figure 1C). On the contrary, quinacrine reduced viability of ScN2a cells by 50% at the concentration of EC_{99} (1.5 μM) (Figure 2C). As previously demonstrated[13,14], quinacrine inhibited PrP^{Sc} formation in ScN2a cells (EC_{50} = 0.45 μM, EC_{99} = 1.5 μM) but less efficiently.

When both rPrP-Q218K and quinacrine were applied onto ScN2a cells simultaneously, an additive inhibitory effect was observed. After the combined administration of 0.5 μM of quinacrine and 0.22 μM (5 μg/ml) of rPrP-Q218K, PrP^{Sc} formation in ScN2a cells was reduced by another 30% compared with quinacrine alone (Figure 2A, 2B). When combined with 0.43 μM (10 μg/ml) of rPrP-Q218K, EC_{99} of quinacrine was also decreased from 1.5 μM to 0.5 μM, in which the cell viability was recovered from 50% to over 90% as inversely proportional to the concentration of quinacrine (Figure 2C).

In order to gain further mechanistic insight into the inhibition of PrP^{Sc} formation by rPrP-Q218K, morphological and biochemical analyses were performed. Indirect immunofluorescent microscopy detected these rPrPs on the cell surface with no difference in distribution profiles (Figure 3). These results show that some factor/s other than the GPI-anchor rendered the rPrPs detected on the cell surface.

SPR measurement revealed that both analytes of rPrP-Q218K and rPrP-WT did not bind to rPrP-WT immobilized onto the chip surface as a ligand, whereas anti-prion mAb 6H4 bound the ligand of rPrP at the equilibrium dissociation constant (K_D) of 2.2 x 10 ^{9}. In order to correct the instrumental noise and non-specific binding, the sensorgram of the flow cell containing rPrP-WT-immobilized sensor chip was subtracted from that of a blank cell. However, SPR could not detect any interaction between amine-coupled rPrP-WT on the surface of the chip and soluble rPrP-Q218K/rPrP-WT in the flow, indicating their KD values below the detection limit of SPR measurement.

Discussion

While mouse Q218K in a GPI-anchored form has already been known as a dominant negative in ScN2a culture cells[6,7] and transgenic mice[8], we have demonstrated that the administration of non-GPI-anchored rPrP-Q218K sufficiently inhibits PrP^{Sc} formation in ScN2a cells for the first time.

Meier *et al.* recently reported that soluble wild-type PrP derivatives might represent a new class of prion replication antagonists with transgenic and gene knockout approaches[15]. In PrP transgenic mice with a wild-type background, the expression of PrP^{C} rendered soluble and dimeric by fusion to immunoglobulin Fcγ (PrP-Fc2) delays PrP^{Sc} accumulation, agent replication, and onset of disease following inoculation with infective prions. While it is preliminary to consider such gene therapeutics, *e.g.* an *ex vivo* gene transfer approach[16,17], the direct administration of soluble rPrPs such as our rPrP-Q218K would be an alternative approach for prion therapeutics. In addition, homogeneous and high-quality soluble rPrP-Q218K could be easily prepared from large-scale fermentation of *E. coli* in sufficient quantities.

Artificial administration of anti-PrP antibodies have been shown to exert a protective effect against infection with PrP^{Sc} [18, 19], which is in good agreement with our own data *in vitro*; EC_{50} of anti-PrP antibody Fab D18 (kindly provided by Dr. Stanley B. Prusiner) = 6 nM, and EC_{99} = 30 nM (data not shown). However, a recent clinical trial of Aβ vaccination targeting Alzheimer's disease has been halted due to the serious neurological complications of autoimmune reactions developing in

FIGURE 3: Both rPrP-WT and rPrP-218K bind to N2a cells. N2a cells are incubated for 3 h with PBS(–), 10 μg/ml rPrP-WT or rPrP-Q218K, washed with PBS(–), fixed with 2% formalin, and subjected to indirect immunofluorescent microscopy. rPrPs, asialo-GM1 are displayed in green and red, respectively. PrP^{C} and asialo-GM1 are localized on the plasma membrane. Both rPrPs are detected on a part of lipid rafts. Bar: 15 μm.

some patients[20,21]. In contrast, the generation of anti-PrP antibodies *in vivo* has proven quite difficult in wild-type animals, PrP being a notoriously poor immunogen[22]. From the aspect of avoiding such unwanted autoimmune reactions, rPrPs might also be considered as a better candidate for prion therapeutics.

Quinacrine, an anti-malarial drug, was reported to inhibit PrP^{Sc} formation in ScN2a cells[13,14], and has already been prescribed for CJD patients in a tentative way[23]. Unfortunately, quinacrine tends to provoke a drug-induced liver dysfunction[24], which frequently forces cessation of the drug administration. In order to minimize the side effects of quinacrine, we expected that combination treatment of multiple anti-prion drugs might be an alternative option. In fact, when combined with rPrP-Q218K, the EC_{99} of quinacrine was successfully reduced and no significant cytotoxicity was observed at the same range of concentration in ScN2a cells. Such a combination could alleviate the side effects of quinacrine by reducing its effective concentration without changing or even accelerating the inhibition efficacy.

Enari *et al.*[25] proposed that sequestration of PrP^{C} by anti-PrP antibody or removal by phosphatidyl-inositol specific phospholipase C (PIPLC) leads to depletion of PrP^{Sc} due to much more rapid turnover of PrP^{Sc} than previously supposed. Since decrease in PrP^{C} by administration of rPrP-Q218K to ScN2a cells was not detected (Figure 1A), the inhibition of PrP^{Sc} accumulation seems to be caused by secession of PrP^{Sc} synthesis, stimulation of PrP^{Sc} degradation or a combination of both.

It was shown that both rPrP-Q218K and rPrP-WT in non-GPI-anchored form were equally detectable on the cell surface, where the conversion of PrP^{C} into PrP^{Sc} takes place[26,27]. Of note, such equal binding seems to be independent of the inhibition of PrP^{Sc} formation, since only rPrP-Q218K but not rPrP-WT exclusively inhibited the PrP^{Sc} formation in ScN2a cells. Nonetheless, one might assume that rPrP-Q218K aberrantly binds to endogenous PrP^{C} in terms of the binding partner, which is undetectable by the conventional immunofluorescent microscopy, and concurrently inhibits PrP^{Sc} formation. Real-time kinetics analysis by SPR, however, failed to detect any significant difference in the binding kinetics of rPrP-WT as a ligand with rPrP-WT or rPrP-Q218K as analytes. Measurements of on-rates (ka) and off-rates (kd) of protein-protein interactions made by SPR is extremely sensitive, and these values are directly related to KD[28]. Taken into account the fact that SPR successfully detects binding interactions in the order of 10^{-3} ~ $^{-4}$ of KD[28], it seems less likely that such discrepancy between rPrP-Q218K and rPrP-WT on the PrP^{Sc} inhibition could be explained by the different binding kinetics of these rPrPs against PrP, although rPrP may not perfectly substitute for PrP^{C}. Instead, it seems likely that these rPrPs more readily interact with a binding factor/s other than PrP where rPrP-Q218K and rPrP-WT are equally detected but inhibit PrP^{Sc} formation differently. Legname *et al.*[29] recently reported that the dominant-negative MoPrP (MoPrP-Q218K)-Fc, in which the C-terminus of MoPrP was fused to the Fc portion of an IgG, not only binds to granule cells but also binds to neurons of the molecular layer where PrP^{C} is expressed, and assuming that the cells of the molecular layer express an auxiliary protein/s, provisionally designated protein X[6,30], which is involved in prion replication. Identification of such factor/s remains to be further examined.

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FULL PAPER *Immunology*

Establishment of a Chicken Monoclonal Antibody Panel Against Mammalian Prion Protein

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ABSTRACT. A panel of chicken monoclonal antibodies (mAbs) was developed against prion protein (PrP), the sequence of which is a highly conserved molecule among mammals. A portion of the splenocytes from chickens immunized with recombinant mouse PrP was fused with the chicken B cell line, MuH1. The remaining splenocytes were used to generate the recombinant mAbs by phage display. A total of 36 anti-PrP mAbs, 2 from cell fusion and 34 from phage display were established. The specificity of these mAbs was determined by Western blot and ELISA using various PrP antigens including recombinant PrPs, synthetic PrP peptides and PrPs from brains or scrapie-infected neuroblastoma cell line. These mAbs were classified into three main groups, protease K (PK)-sensitive (Group I), PK cleavage site proximal (Group II) and PK-resistant (Group III), based on their abilities to recognize PrP following PK-treatment. Some mAbs were found to selectively recognize different glycoforms of PrP as well as the metabolic fragments of PrP. Furthermore, we found that PrP recognition by chickens differed from that by PrP-knockout mouse. These results indicate that these newly generated PrP antibodies from chickens will help to research the PrP and to establish the diagnosis of prion disease.

KEY WORDS: avian, panel mAb, prion protein.

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Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders such as Creutzfeldt-Jacob disease (CJD) in human, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) [15]. These disorders are believed to occur through the accumulation of an abnormal, pathogenic isoform (PrP^{Sc}) of cellular prion protein (PrP^{C}). Although PrP^{C} and PrP^{Sc} contain identical amino acid sequences [18], they have quite different biochemical properties. PrP^{C} is soluble in most detergent and is completely digested by proteases, whereas the pathogenic PrP^{Sc} is insoluble in detergents and is partially resistant to proteases [7, 11, 14]. In addition, PrP^{C} is rich in α-helices, whereas PrP^{Sc} is a β-sheet-rich structure [12]. Despite this knowledge, the mechanisms by which conversion from PrP^{C} to PrP^{Sc} occurs remain unclear.

The final confirmatory diagnosis of TSE and the study of basic prion biology and disease pathogenesis require the development of sensitive PrP-specific monoclonal antibodies (mAbs) [17, 23]. Development of mAbs against PrP with mouse hybridoma technology has brought about enormous progress in biological as well as diagnostic studies [3]. However, it is not easy to generate specific mAb against PrP molecules without the use of PrP-knockout mice since the homology among mammalian PrP amino acid sequences is more than 84% [11]. Conversely, chickens are located on a different branch of the phylogenetic tree from mammals, and avian PrP has low homology (less than 40%) with mammalian PrP, suggesting that these birds may be ideal hosts for generation of specific antibodies to mammalian PrP [2, 5, 6].

The molecular diversification of immunoglobulin (Ig) in the chicken differs from that in mammals. In mammals, the primary B lymphocyte repertoire is generated by V(D)J rearrangements involving the Ig variable region gene segments of the Ig heavy and light chains [1, 16]. It is possible to perform RT-PCR of the V-region repertoire with a single pair of primers making chicken antibody extremely useful for antibody engineering. Recently, we developed chicken mAbs against mammalian PrP by cell fusion technology [5, 6]. We constructed a phage-display of chicken hybridoma HUC2–13 [5] in order to produce a large amount of the mAb [8]. In another report, a panel of chicken antisera against synthetic sheep PrP peptides has been developed [2].

To generate a panel of chicken mAbs to mammalian PrP for application in prion research, we utilized two technologies, cell fusion and phage-display, using chicken immunized with recombinant mouse PrP. All chicken mAbs cross-reacted with a broad range of mammalian PrP sequences. Specific recognition of PrP by some of the antibodies was affected by the degree of PrP glycosylation. Furthermore, some mAbs were highly reactive against PK-treated PrP^{Sc}. We herein describe the establishment of a panel of chicken mAbs against mammalian PrP and discuss the possibility of their use for basic and applied prion research.

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MATERIALS AND METHODS

Cell lines: The chicken B cell line, MuH1 [10], was used as the fusion partner. MuH1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 38.5°C. The scrapie-infected mouse neuroblastoma cell line, N2a/22L [9], (kindly supplied from Dr. Nishida of Nagasaki Univ., Japan), was used as a source of PrP^{Sc}. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS in a 5% CO_2 incubator at 37.0°C.

Antigens: Recombinant PrP fragments, M23–231 and M23–87 (mouse PrP: codon 23–231 and 23–87) were kindly supplied by Dr. Shinagawa (Obihiro Univ. Agric. Vet. Med., Japan). Recombinant PrP fragments, H23–230 and H122–230 (human PrP: codon 23–230 and 122–230), were kindly supplied by Dr. Kitamoto (Tohoku Univ., Japan). Three recombinant PrP fragments having histidine tag, M121–231 (mouse PrP: codon 121–231), S125–234 (sheep PrP: codon 125–234) and B133–241 (bovine PrP: codon 133–241), were generated with pET22b (Novagen, U.S.A.), and purified with nickel ion-charged Chelating Sepharose Fast Flow (Amersham Biosciences, Sweden) and HiPrep Sephacryl S-100 HR (Amersham Biosciences, Sweden) according to manufacturers' instructions. Synthetic mouse and human PrP peptides (M53–68, M69–84, M85–100, M94–109, M109–127, M124–144, M141–159, M156–175, M172–181, M182–190, M187–204, M205–224, M220–242 and H25–49) were synthesized by a solid-phase peptide synthesizer system (Model PSSM-8, Shimazu, Japan). M23–231 and M121–231 were used as immunogens.

Immunization and cell fusion: One-month-old H-B15 inbred chickens were immunized intraperitonealy (i.p.) with recombinant mouse PrPs (100 μg/0.5 m*l*/chicken) in an equal volume of Freund's complete adjuvant. The chickens received three additional i.p. injections of the corresponding antigen in Freund's incomplete adjuvant at 3-week intervals. Three days after the final boost MuH1 cells were fused splenocytes from a chicken immunized with M23–231. Cell fusion experiments were performed by the method described previously [5].

RT-PCR: Total RNA was extracted with ISOGEN-LS (Nippon Gene, Japan) from the splenocytes from a chicken immunized with M23–231 that were not used in cell fusion (1.0×10^9 cells), and splenocytes (1.0×10^9 cells) from a chicken immunized with M121–231. First strand cDNA was primed with Oligo-$(dT)_{12-18}$ primer (Roche Diagnostics, Switzerland) and synthesized with Superscript II synthesis cDNA Kit (GIBCO BRL, U.S.A.). The V-regions of the antibodies were amplified by PCR with KOD plus DNA polymerase (TOYOBO, Japan) using two primer pairs: CHB (forward) and CHSF (reverse) for amplification of heavy chains (VH) and CLSB (forward) and CLF (reverse) for variable light chain (VL) [8]. The scFv linker was prepared by PCR of pLINK (Accession number: D50400) [20, 21]. In order to make the scFv (single chain fragment of variable region) fragment, purified VH and VL fragments were assembled with purified scFv linker by PCR. The reamplification of scFv was performed using CRB (forward) and CRF (reverse) [8].

Construction of phage display library: Two hundred *n*g of scFv fragments was ligated with 1 μg of pPDS (Accession number: D50401) [20, 21] at the *Eag*I and *Bss*HII sites. The resultant DNA was electroporated in five portions into 80 μ*l* aliquots of Epicurian Coli XL1-Blue Electroporation Competent Cells (Stratagene, U.S.A.). After incubation for 1 hr in LB medium (10 g of trypton, 5 g of yeast extract, and 10 g of NaCl per litter), an aliquot was taken to titrate the library size on LB agar plates. The culture was then infected with VCS-M13 (7.5×10^9 PFU, Stratagene, U.S.A.) in medium containing ampicillin (100 μg/m*l*), tetracycline (50 μg/m*l*) and 1% glucose. The cells were collected by centrifugation at 800 × g and resuspended in 100 m*l* of SuperBroth containing ampicillin (100 μg/m*l*), tetracycline (25 μg/m*l*), and kanamycin (50 μg/m*l*). The culture was incubated overnight at 37°C with shaking vigorously. The phage display library containing supernatant was filtered through a 0.45 μm pore filter and stored at 4°C.

Panning selection for PrP-specific antibodies: The phage-display scFv library from M23–231-immunized chicken (library A) was panned against M23–231 and M121–231, whereas, the library from M121–231-immunized chicken (library B) was panned against M121–231. For their selection, 50 μ*l* (5 μg/m*l*) of antigens was coated on a 96-well microtiter plate (Maxisorp Nunc-Immuno™ module, Nunc, U.S.A.). The panning was performed by the method described previously [8].

Sequencing of phage-display antibodies: After the second or third panning, the phage-displayed antibodies (60 clones from library A and 80 clones from library B) were classified based on the Ig gene sequences. The determination of Ig genes was performed by using BigDye terminator cycle sequencing kit (Applied Biosystems, U.S.A.) and 3100 Genetic Analyzer (Applied Biosystems, U.S.A.).

ELISA for reactivity and epitope analysis: The wells of ELISA plates were coated with 50 μ*l* (2.5 μg/m*l*) of recombinant PrPs and synthetic peptides, and blocked for 2 hr at 37°C with 25% BlockAce in PBS. These antigens were used in non-reduced conditions. The mAbs from hybridoma and the recombinant mAbs (4.0×10^{10} CFU/m*l*) were then added to each well of the plate and incubated at 37°C for 1 hr. The bound mAbs were detected by the method described previously [8].

Western blot detection of PrP^C and PrP^{Sc}: Normal brain tissue from Balb/c mice, sheep and bovine, respectively, were homogenized in 9 vol of lysis buffer (10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% Triton-X100, 0.5% sodium deoxycholate) plus a protease inhibitor cocktail (Roche Diagnostics, Switzerland). Homogenates were centrifuged at 800 × g for 5 min. Protein concentration in the supernatant was measured with the DC protein assay kit (BIO-RAD, U.S.A.). Mouse PrP^{Sc} was prepared from scrapie-infected mouse brain (kindly supplied from Dr. Yokoyama of NIAH, Japan) and a scrapie-infected mouse

neuroblastoma cell line N2a/22L. The preparation of PK-treated PrP^{Sc} from brain and N2a/22L was performed as described previously [9, 22]. The SDS-PAGE in non-reduced condition and Western blotting was performed as described previously [8].

RESULTS

Generation of chicken monoclonal antibodies to PrP by cell fusion: MuH1 cells were hybridized with spleen cells from a chicken immunized with M23–231. Two hybridoma clones producing specific antibody were identified. The mAbs, named HUNN1 and HUNN2, were both classified as IgG (IgY) (data not shown).

Selection of PrP-specific antibodies from a phage-display library: cDNAs generated from the variable region of chicken Ig mRNA, were approximately 380 bp for the heavy chain and 340 bp for the light chain. The cDNAs were amplified (data not shown) and assembled into single chain fragment of variable region (scFv) fragments via a linker sequence. The sizes of the scFv libraries were 2.7×10^7 CFU/μg vector (library A) from an M23–231-immunized chicken and 1.0×10^7 CFU/μg vector (library B) from an M121–231-immunized chicken. The recombinant phages ($0.1–1.0 \times 10^{12}$ CFU/m*l*) were panned against specific antigen. The number of bound phages eluted with acidic buffer showed a successive increase in each round of panning (data not shown). The reactivity of bound phages in each panning also progressively increased (Fig. 1). However, the phage display antibodies against M121–231 from library A could not be developed.

Characterization and reactivity of monoclonal phage-display antibodies: Phage-display mAbs (60 clones from library A and 80 clones from library B) were subjected to DNA sequencing. Consequently, four (phAb1–4, phAb2–1, phAb3–7 and phAb3–15) from library A and 30 (phAb4–1 to –30) from library B were identified. These antibodies were constituted from the combination of 24 heavy chains and 26 light chains arrangement in amino acid level (data not shown). Figure 2 shows the amino acid sequences of both heavy and light chains of 4 clones (phAb1–4, phAb2–1, phAb3–7 and phAb3–15) in library A and 3 clones (phAb4–3, phAb4–12 and phAb4–19), with the high incidence in library B. The light chain sequences of phAb1–4 and phAb2–1 were identical, although their heavy chain sequences varied significantly. The sequence of each heavy and light chain CDR from phAb4–3, phAb4–12 and phAb4–19 was nearly identical, varying maximally at four residues. Notably, the heavy chain CDR3s of these three mAbs were identical. The homology between heavy and light chain CDRs of phAb3–7 and phAb3–15 were also high. The CDR sequences of the remaining 27 clones were similar to those of phAb4–3, phAb4–12 and phAb4–19 (data not shown). The seven mAbs described above were chosen for use mainly in subsequent experiments.

The reactivity of the phage-displayed mAbs to PrP was examined by ELISA (Table 1). The HUNN1, HUNN2,

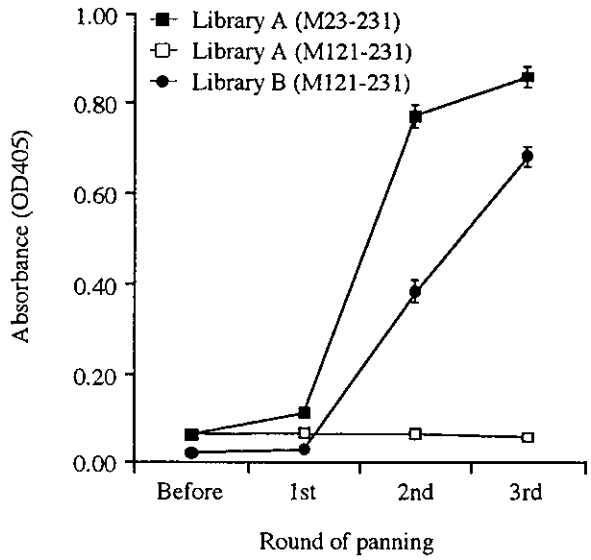

Fig. 1. ELISA reactivity of phage-displayed antibodies after the each round of panning against antigen. The phage-displayed antibodies (4.0×10^{10} CFU/m*l*) from libraries A and B were examined by using recombinant MoPrPs, M23–231 and M121–231, respectively. Data represent mean ± SD, n=3 wells/experiment.

phAb1–4, phAb2–1, phAb3–7 and phAb3–15 from M23–231-immnized chicken showed reactivity against M23–231 and H23–231, but not for M121–231 and H122–230. The HUNN1, HUNN2, phAb3–7 and phAb3–15 reacted with M85–100 and M94–109 mainly and the phAb3–15 reacted also with M124–144 slightly. The phAb1–4 and phAb2–1 reacted with M23–87. The three mAbs from library B (phAb4–3, phAb4–12 and phAb4–19) were cross-reactive to mammalian PrP (M121–231, H122–230, S125–234 and B133–241). However these 3 mAbs and remaining 27 mAbs from library B were not reacted with the other PrP peptides.

Western blotting for PrP^C from various animals: The reactivity of chicken mAbs against PrP^C from brains was determined by Western blotting. The mAbs HUNN1 and HUNN2 from hybridomas recognized the full-length form of PrP^C (Fig. 3A). HUNN1 reacted strongly with mouse and bovine PrP, but weakly with sheep PrP. In addition, HUNN1 reacted strongly with the di-glycosylated form of PrP^C. The pattern of HUNN2 reactivity to PrP was similar to that of the HUNN1, however, recognition of sheep PrP was lower than that of HUNN1. Both mAbs also reacted strongly with PK-treated PrP^{Sc} as described below. As a negative control, neither of reacted with brain homogenate from the PrP gene knockout (*Prnp*$^{0/0}$) mouse (data not shown).

The phage-display mAbs, phAb1–4 and phAb2–1, weakly recognized mouse PrP (Fig. 3B) despite indications of high reactivity by ELISA (Table 1). The reactivities of phAb1–4 and phAb2–1 differed but for both reactivity to

Heavy chains

```
                                                  CDR1                    CDR2
germline H     1 AVTLDESGGGLQTPGRALSLVCKASGFTFSSYNMGWVRQAPGKGLEFVAGIDNTGRYTGY    60
1-4H           1 ----------------G-----------D----A-N-----------W----SAA--:-T-    59
2-1H           1 ----------------G--------G-------F--F--------------EVSGD-S--Y-    60
3-7H           1 ----------------GG------G-R-D---F--A----------------SSD-SG---    60
3-15H          1 ----------------GG----------D---F--A----------------SSD-SG-A-    60
4-3H           1 ----------------G-------------ND-G-N--------------Q-SSS-GS-Y-    60
4-12H          1 ----------------GG------G------D-G-N--------------Q-SS--SS-W-    60
4-19H          1 ----------------G-----------D----G-N--------------Q-SS--SS-Y-    60

                                                                  CDR3
germline H    61 GSAVKGRATISRDNGQSTVRLQLNNLRAEDTGTYYCAKAAG:XXXXXXXXXXTAGSIDAW   119
1-4H          60 VA--D--------D-----------------AI-F--:::::RNRNNVYYYNTAW----   113
2-1H          61 AP----------A-----M-------------------G--:::::SGYYYN-VNE--T-   115
3-7H          61 AP--E----------NIL-------------A------RT--SDVASSSRCGH-G------   120
3-15H         61 VP--T--------------------------A------T--SGDGSTTTCGHIG------   120
4-3H          61 AP----------D-----------------A------T--G:::::SGYSR---Y----   115
4-12H         61 AP-------------------------------AN-----T--G:::::SGYGR---Y--T-   115
4-19H         61 AP-------------------------------A------T--G:::::SGYSR---Y----   115

germline H   120 GHGTEVIVSS                                                    129
1-4H         114 ---------T                                                    123
2-1H         116 ---------T                                                    125
3-7H         121 ---------T                                                    130
3-15H        121 ---------T                                                    130
4-3H         116 ---------T                                                    125
4-12H        116 ---------T                                                    125
4-19H        116 ---------T                                                    125
```

Lambda chains

```
                                        CDR1                    CDR2
germline L     1   ALTQPSSVSANPGGTVKITCS:GDS:::S:YYGWYQQKAPGSAPVTVIYDNTNRPSNI    53
1-4L           1 DV-----A---T---E-------G-G-YAG-YY---F--------L--L--N-N----D-    60
2-1L           1 DV-----A---T---E-------G-G-YAG-YY---F--------L--L--N-N----D-    60
3-7L           1 DV-----A---T-L---------GNSGC:G:::----R--S-------L--N-DQ---D-    56
3-15L          1 DV-----A-----L----R----GTSGC:G:::-------S----------N-DK---D-    56
4-3L           1 DV-----A-------E-------G-GTYAGNYY-------T------S---GS-S-----    60
4-12L          1 DV-----A-------E-------G-GTYAG-YY-------S----------GS-S-----    60
4-19L          1 DV-----A-------E-------G-GTYAGNYY--------------S---GS-S-----    60

                                                CDR3
germline L    54 PSRFSGSKSGSTATLTITGVRADDNAVYYCASTD:SSSTAGIFGAGTTLTVLG          105
1-4L          61 ------------N-------Q---E-----G-A-:--G:-------------K          111
2-1L          61 ------------N-------Q---E-----G-A-:--G:-------------K          111
3-7L          57 -------A------------QVE-E------NF-SDTKFSDT----------K          109
3-15L         57 -------T----N---------E-E------NF-SDTELTDT----------K          109
4-3L          61 ----------------------E-E-I---G-A-:D-THV------------K          112
4-12L         61 -------A--------------E-E-----G-A::D-IDV-M----------K          111
4-19L         61 ----------------------E-E-I---G-A-:D-THV------------K          112
```

Fig. 2. Amino acid sequences of anti-PrP phage-display mAbs. Germline sequences of CB inbred strain were shown at the top line. CDRs were underlined. Amino acid identity to the germline sequences was shown by dash. The absence of corresponding residues was shown by colons. Amino acid residues from D segment gene in the heavy chain were shown by an X.

Table 1. Summary of PrP-specific chicken mAbs

Antigen	Antibody									
	HUC2–13[a]	phAb1–4	phAb2–1	HUNN1	HUNN2	phAb3–7	phAb3–15	phAb4–3	phAb4–12	phAb4–19
	Reactivity[b] in ELISA									
M23–231	+	+	+	+	+	+	+	+	+	+
H23–230	+	+	+	+	+	+	+	+	+	+
M121–231	–	–	–	–	–	–	–	++	++	++
H122–230	–	–	–	–	–	–	–	++	++	++
S125–234	–	–	–	–	–	–	–	+	+	+
B133–241	–	–	–	–	–	–	–	+	+	+
M23–87	+	++	++	–	–	–	–	–	–	–
M85–100	–	–	–	++	+	++	+	–	–	–
M94–109	–	–	–	+	+	+	++	–	–	–
M124–144	–	–	–	–	–	–	±	–	–	–
H25–49	++	–	–	–	–	–	–	–	–	–
Brain	Reactivity in Western blotting									
M[c]	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	–	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+
	Grouping of mAbs									
	◀—— Group I ——▶			◀—— Group II ——▶				◀—— Group III ——▶		

a) The mAb has been established previously [5].
b) ++, strong reactive (OD value: >1.5); +, moderate reactive (OD value: 0.5 to 1.5); ±, low reactive (OD value; 0.1 to 0.5); –, non-reactive (OD value: <0.1).
c) M, mouse; S, sheep; B, bovine.

sheep and bovine PrPs was higher than the reactivity to mouse PrP. The phAb3–7 and phAb3–15 recognized mouse, sheep and bovine PrPs although recognition varied with the degree of PrP glycosylation. The phAb4–3, phAb4–12 and phAb4–19, consistently failed to react with di-glycosylated PrPs but recognized all other forms, including the truncated forms reported previously [17,23] (Fig. 3C). Moreover, the reactivities of these mAbs against PrPs decreased under the reduced-condition (data not shown). The detection patterns of the remaining 27 mAbs from library B were also identified (data not shown).

Western blotting of mouse PrP^{Sc}: To examine whether chicken mAbs detect PK-treated PrP^{Sc}, Western blotting was performed with the homogenate of a scrapie-infected mouse brain and a scrapie-infected neuroblastoma cell line N2a/22L (Fig. 4). HUNN1, HUNN2, phAb3–7 and phAb3–15 reacted equally with all three glycoforms of PK-treated PrP^{Sc}, however phAb4–3 and phAb4–19, recognized the mono- and un-glycosylated forms of PrP^{Sc}, but failed to recognize the di-glycosylated form. In addition, phAb4–12 reacted with mono- and un-glycosylated forms of PrP^{Sc} from infected mouse brain and N2a/22L cells, and also with the di-glycosylated form of PrP^{Sc} from infected mouse brain but not with PrP^{Sc} from the N2a/22L. The phAb1–4 and phAb2–1 did not react with PK-treated PrP^{Sc}.

Establishment of chicken mAb panel: The chicken mAbs established in this study as a panel mAb specific for PrP were classified into three main groups, protease K (PK)-sensitive (Group I; 23–90 amino acid residues), PK cleavage site proximal (Group II; 90–110 residues) and PK-resistant (Group III; 121–123 residues), based on their abilities to recognize PrP following PK-treatment, although some mAbs could not be exactly clarified in their antigenic epitopes (Table 1). Chicken mAb HUC2–13 [5] reactive for N-terminal of mature PrP was added in the list of this panel mAb.

DISCUSSION

Immunological detection is the most efficient and reliable diagnostic method for identifying prion disease in human or in domestic animals. Due to high homology (>84%) between mouse PrP and that of other mammalian species, it is advantageous to use the $Prnp^{0/0}$ mouse for production specific mAbs against mammalian PrP. Mouse mAbs specific for intact forms of PrP^{C} [22] and PrP^{Sc} [4] have been established by using the $Prnp^{0/0}$ mice. However, it was reported that hybridomas derived from $Prnp^{0/0}$ mice either stopped secreting PrP-specific mAbs or died soon after their creation [19]. On the other hand, in the chicken mAb system, we were able to produce chicken mAbs against synthetic PrP fragments [5,6]. In this study, we immunized chickens with recombinant mouse PrP (MoPrP), and tried to establish a panel of mAb using both cell fusion and phage-display systems. The mAbs were able to detect PrP in Western blots of healthy brains (mouse, sheep and bovine), infected brain (mouse) and a neuroblastoma cell line, N2a/22L, representing a new tool in prion research.

The mAbs raised in this study were directed against distinct epitopes throughout the 23–231 PrP fragment. These

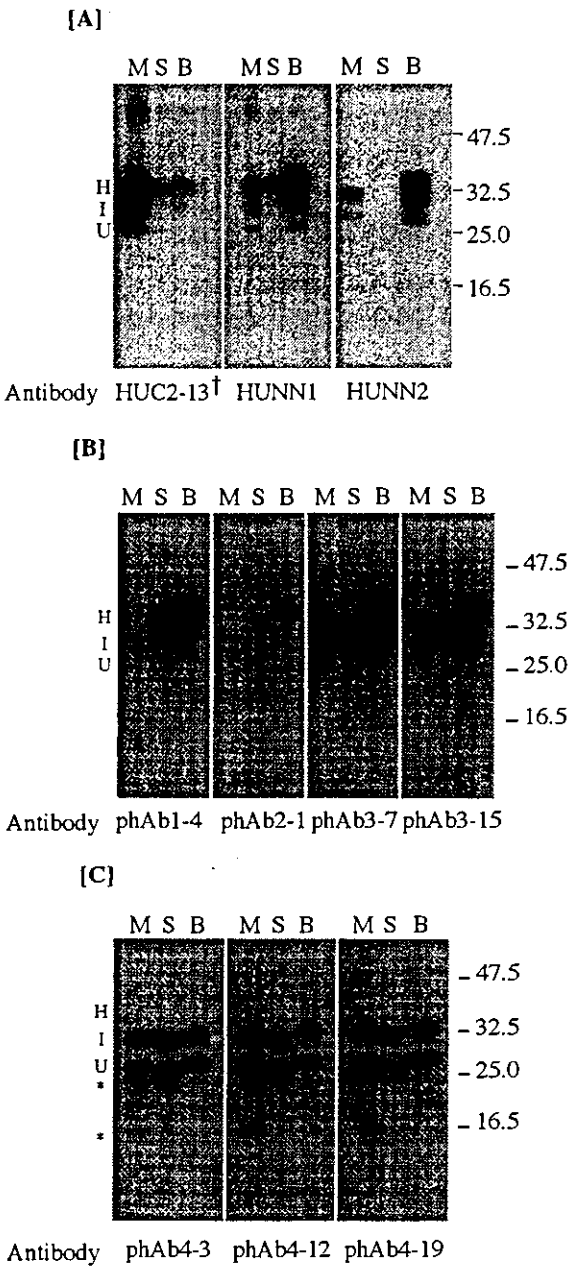

Fig. 3. Western blot analysis of mammalian PrP^C using mAbs from cell fusion and phage display antibody libraries. Ten percent homogenates of healthy mouse (M), sheep (S) and bovine (B) brains were analyzed by Western blot using the mAbs derived from cell fusion [A], library A [B] and library B [C]. HUC2–13 was used as a positive control for detection of PrP. H, I and U represent di-glycosylated, mono-glycosylated and non-glycosylated PrP^C, respectively. * represents truncated PrP^C. The numbers at right indicate apparent molecular masses in kDa. † The HUC2–13 mAb was characterized previously (Matsuda *et al.*, 1999).

mAbs were largely divided into 3 groups, PK-sensitive (Group I), PK-cleavage site-proximal [13] (Group II) and PK-resistant (Group III) based on their ability to recognize PK-treated PrP. Group III was subdivided into two groups based on detection patterns upon Western blot analysis, that is phAb4–12 reacted with 3 forms of PK-treated mouse PrP^{Sc} but phAb4–3 and phAb4–19 reacted with 2 forms of the PrP^{Sc} other than di-glycosylated form (Fig. 4). The Group I mAbs (phAb1–4 and phAb2–1) appear to recognize the octapeptide repeats domain in the PK-sensitive region, since they reacted to M23–87 but not to H25–49 and PK-resistant PrP. The HUC2–13 [5] reactive for H25–49 was also added in this Group I mAbs. The Group II mAbs (HUNN1, HUNN2, phAb3–7 and phAb3–15) were generated from cell fusion (2 of 2 clones) and library A (2 of 4 clones). This result suggests that the region (PK-cleavage site-proximal) of recombinant MoPrP may have powerful antigenicity in chickens though the reasons were unknown. In fact, it has been reported that the antigenicity of this region as well as the octapeptide repeat is high for chickens [2]. The Group III mAbs (phAb4–3, phAb4–12 and phAb4–19) preferentially recognized the mono- and un-glycosylated forms but not the di-glycosylated form of PrP^C. This detection pattern of Group III mAbs was similar to PrP-specific mouse mAb 6G9 that recognized an epitope that resides in or near the N-glycosylation site of MoPrP residue 180 [23]. However, Group III mAbs did not react PrP peptides including N-glycosylation site and the other peptides of PrP. These mAbs may be recognized the conformational epitopes formed of disulfide bond, because these mAbs hardly reacted PrPs from brains under the reduced-condition. Since the detection patterns of the remaining 27 clones from library B were similar to that of Group III mAbs, the epitopes recognized by these mAbs are also conformational one. The classification of all the phage-displayed mAbs based on the identity of CDR sequences (amino acid level) in the heavy and light chains was completely in agreement with the classification of the mAbs by the antigen reactivity.

Although mAbs derived from the M23–231-immunized chicken were classified as Group I or II, none recognized the C-terminus of PrP. Conversely, most antibodies from the M23–231-immunized $Prnp^{0/0}$ mouse recognized a C-terminal region of the protein (codon 145–220) [23]. These results may reflect differences in prion protein recognition by chicken and mouse. If such differences were present, the use of both animals, would allow the creation of a panel of antibodies that can cover all prion protein regions.

It is expected that the phage-display antibodies potentially possess recognition of conformational epitopes, because the antibody consists of an artificial combination of heavy and light chain genes from various B cells. Therefore, the phage display system may be a good method for making antibodies to conformational epitopes of PrP^{Sc}.

In this study, we demonstrated that mAb-production in chickens is a potentially fruitful alternative to murine mAb production for generation of mAbs against mammalian PrP. The chicken antibody library offered a range of PrP-specific

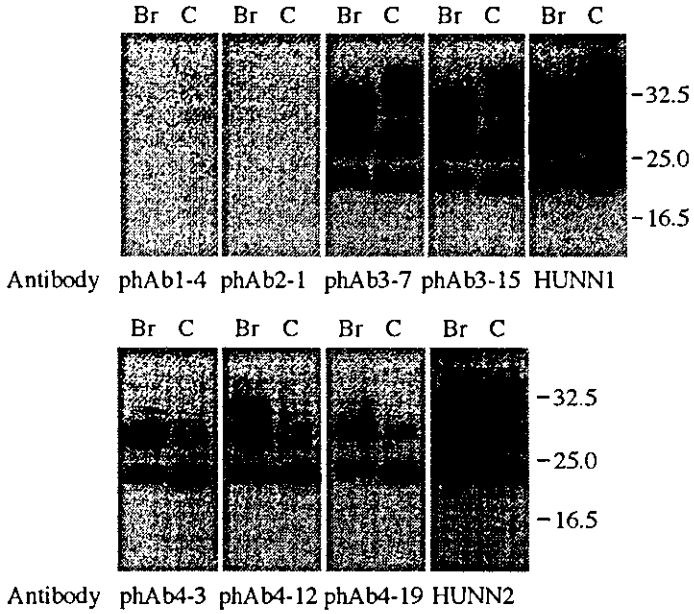

Fig. 4. Western blot analysis of mouse PrP^{Sc} using chicken mAbs. PrP^{Sc} prepared from scrapie-infected mouse brain (Br) and N2a/22L cell line (C) was analyzed by Western blot using chicken mAbs derived from cell fusion and phage-displayed antibody library. The numbers at left indicate apparent molecular masses in kDa.

antibodies similar to the variety produced using $Prnp^{0/0}$ mice. The methodological improvement of using the intact form of PrP^{C} and PrP^{Sc} molecules to screen antibodies, will allow the chicken mAb system to contribute greatly to the progression of basic and diagnostic research on prion disease.

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Expression vectors for chicken–human chimeric antibodies

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Abstract

The chicken is a useful animal for preparation of antibodies that are reactive with highly conserved mammalian molecules. For further clinical application of chicken antibodies, we constructed the novel expression vectors for chicken–human chimeric antibodies, pcSLCγ1, pcSLCγ4 and pcSLCκ. These vectors had the following characteristics: (1) any chicken variable regions from hybridomas or a phage display library can be easily introduced; (2) the variable regions are able to be expressed in different immunoglobulin isotypes; and (3) the chimeric antibodies can be highly expressed in either transiently or stably transfected eukaryotic cells (COS-7 and CHO-K1 cells). Western blot analysis of the chimeric antibodies revealed that the expressed products were of the predicted size, structure and specificity. These results indicate that these vectors are useful tools for the chimerization of chicken antibodies.

1. Introduction

Hybridoma technology [1] has yielded numerous useful monoclonal antibodies for both research and diagnostic applications. A large number of targets that possess clinical potential are highly conserved in mammalian species, and therefore, provide limited immune response in rodents due to immunotolerance. An alternative approach to antibody production is the use of non-mammalian species as immunological hosts. The chicken is a useful animal for obtaining antibodies specific for conserved mammalian molecules [2–7] because it is located on a different branch of the phylogenic trees from mammals and has a higher potential for antibody production. Moreover, chicken developed different types of DNA recombination, gene conversion, to generate a large, diverse antibody repertoire [8]. Because both the heavy and light chain loci in chickens consist of single functional variable (V) and joining (J) region genes, it is possible to perform RT-PCR of the V region repertoire with a single pair of primers. Therefore, chicken is extremely useful for antibody engineering.

However, the clinical applications of chicken antibodies are limited. Because chicken antibodies are immunogenic to human, they induce anti-chicken immunoglobulin (Ig) response. To overcome this problem, the most immunogenic regions of chicken Igs (the constant regions) can be replaced with those of humans. Such antibodies are known as chimeric antibodies. Numerous investigators have constructed mouse–human chimeric antibodies [9–14] and have reported decreased immunogenicity and longer serum half-lives [15]. In addition, chimeric antibodies have proven useful for clinical applications [16].

We present here the novel expression vectors for rapid production of chicken–human chimeric antibodies. We tested these vectors both transient and

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stable transfections, and demonstrate the usefulness of this chicken–human chimeric antibody expression system.

2. Materials and methods

2.1. Construction of expression vectors pcSLCγ1, pcSLCγ4 and pcSLCκ

Genomic DNA was prepared from peripheral blood mononuclear cells, which were isolated from the heparinized blood of a healthy volunteer. The human heavy chain constant region genes (Cγ1 and Cγ4) and light chain constant region genes (Cκ) were amplified from genomic DNA by PCR using the primers IgGF and IgGR for heavy chain, and IgKF and IgKR for light chain (Table 1). Purified PCR products were digested with *Eco*RI and ligated into an intermediate vector and verified by sequencing. The *Eco*RI digested 1.8-kb fragments containing human Cγ1 were introduced into the *Eco*RI site of pcDNA4/*myc*-His A (Invitrogen, USA). Constructs containing inserts having the correct orientation were selected by sequencing and named pcDCγ1. Construction of pcDCγ4 was accomplished in the same way as pcDCγ1, except that the restriction site *Not*I was used. The *Eco*RI/*Xho*I digested 0.6-kb fragments containing human Cκ genes were introduced into the *Eco*RI/*Xho*I site of pcDNA3/*myc*-His A (Invitrogen, USA) (pcDCκ).

The chicken heavy chain leader sequence was amplified by PCR from genomic DNA, which was prepared from the chicken hybridoma HUC2-13 [6], using the primers VH3F and VH-LeR. Purified PCR products were digested with *Hin*dIII and *Kpn*I, and ligated into an intermediate vector and verified by sequencing. The *Hin*dIII/*Kpn*I digested fragments containing chicken leader sequence were inserted into the *Hin*dIII/*Kpn*I site of pcDCγ1, pcDCγ4 and pcDCκ in order to obtain expression vector pcSLCγ1 and pcSLCγ4 and pcSLCκ, respectively.

Table 1
PCR primers used in this study

Primer	Sequence
IgGF	5′-TTGAATTCCTTTCTGGGGCAGGCCAGGCCT
IgGR	5′-GCACTCATTTACCCGGAGACAGGGA
IgKF	5′-TTGAATTCCAGAATGGCTGCAAAGAGCTCC
IgKR	5′-CTCTAACACTCTCCCCTGTTGAAGCT
VH3F	5′-ATATATAAGCTTACCATGAGCCCACTCGTCTCCTC
VH-LeR	5′-TTGGTACCGGCGCGCCCTGTGGGGAAGGACA-GAAAGCG
VH4R	5′-TTGGATCCTC*A*CCTGTCGACACGATGACTTC
VH5F	5′-TTGGCGCGCCGCCGTGACGTTGGACGAGTCCGG
VL3F	5′-ATATATAAGCTTACCATGAGCCCACTCGTCTCCTC
VL4R	5′-TTGGATCCACTC*A*CCTAGGACGGTCAGGGTTGT
VL5F	5′-TTGGCGCGCCGCGCTGACTCAGCCGTCCTCGGTG

The restriction sites are underlined. A Kozak sequence is shown in bold and a splice donor signals in italic.

2.2. Cloning of chicken V region genes in expression vector

Chicken V region genes were cloned from HUNN1 hybridoma, and phAb3-15, a single chain fragment of variable region (scFv) selected from a phage display library [7]. Both derived antibodies reacted with prion protein (PrP). Total RNA was extracted from HUNN1 cells with ISOGEN-LS (Nippon Gene, Japan). The first cDNA strand was primed with Oligo-$(dT)_{12-18}$ primer (Roche Diagnostics, Switzerland) and synthesized with a Superscript II Synthesis cDNA Kit (GIBCO BRL, USA). The V region genes were amplified by PCR using the primers VH3F and VH4R for the heavy chain, and VL3F and VL4R for the light chain. Using phAb3-15 as the templates, PCR were performed with VH5F and VH4R for the H chain, VL5F and VL4R for the light chain. Amplified variable genes were first inserted into intermediate vectors for sequencing and were subsequently inserted into expression vectors. *Hin*dIII/*Bam*HI or *Asc*I/*Bam*HI sites were used for insertion.

2.3. Transfection, selection and estimation of antibody production

COS-7 and CHO-K1 cell lines were used for transfection. COS-7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). CHO-K1 cells (Invitrogen, USA) were maintained in F-12 Ham's medium (Invitrogen, USA) supplemented with 10% FBS.

Antibody production was tested for both transient and stable expression. For transient expression, light chain and heavy chain expression vectors were co-transfected into COS-7 cells using Polyfect transfection reagent (Qiagen, USA) according to the manufacturer's instructions. After 72 h of incubation, the culture supernatants were harvested to estimate antibody production. For stable expression, both vectors were co-transfected into CHO-K1 cells using the same methods. After 48 h of incubation, cells were harvested and resuspended in selective medium containing Zeocin (200 μg/ml; Invitrogen, USA) and Geneticin (400 μg/ml; Sigma, USA). Cells were cultured for 2 weeks in selective medium, which was changed every 3–4 days. Resistant cells were harvested, resuspended in a medium containing half the concentration of antibiotics and plated onto 96-well culture plates at <1 cell per well. After 2 weeks, clones were screened for antibody production by ELISA. Positive clones, which reacted with PrP, were selected and further cultured. To estimate antibody production, the culture supernatants were harvested from 10^6 cells cultured for 24 h.