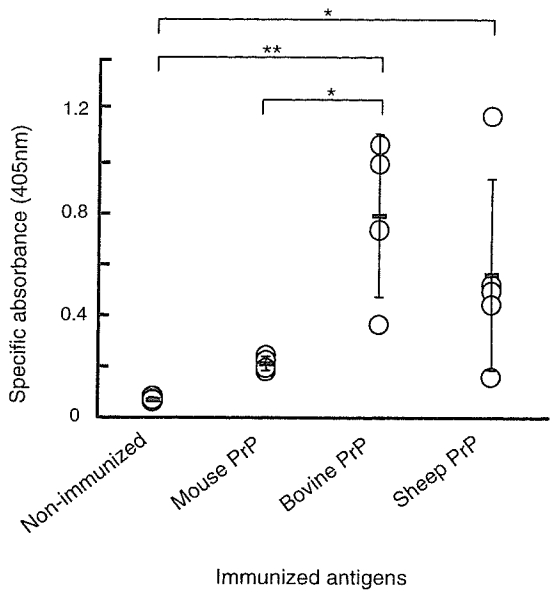

Fig. 3. Anti-PrP autoantibodies in mice immunized with recombinant mouse, bovine, and sheep PrPs. Each antiserum from four to five mice immunized with respective recombinant PrPs was diluted 1:100 and subjected to ELISA against purified mouse recombinant PrP without a 6× His tag. $^{*}p<0.05$; $^{**}p<0.01$.

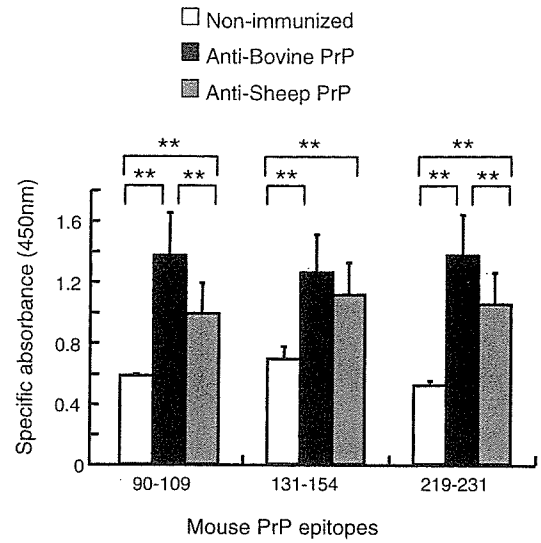

Fig. 4. Recognition of three different mouse PrP epitopes by antisera raised against bovine and sheep recombinant PrPs. The antisera used were collected from four to five mice of each immunization group. Mouse PrP peptides, moPrP90–109, moPrP131–154, and moPrP219–231, positively reacted with antisera raised against bovine and sheep recombinant PrPs on ELISA, compared with sera from non-immunized normal mice ($^{**}p<0.01$).

against the tag with antibodies that might be produced in the immunized mice, we used recombinant mouse PrP without the tag as an ELISA antigen. The antisera raised against mouse recombinant PrP showed only slightly higher OD_{405} values depicting antibody responses at a 100-fold dilution, compared with those of non-immunized mice (Fig. 3). In contrast, a range of much stronger antibody responses showing as higher OD_{405} values was detected with the antisera against bovine recombinant PrP (Fig. 3). The antisera against sheep recombinant PrP showed more variable titers of anti-PrP autoantibodies (Fig. 3). One mouse elicited the highest titer of anti-PrP autoantibodies among the immunized mice, but another mouse exhibited a very weak antibody response showing OD_{405} values as low as those from mouse recombinant PrP-immunized mice (Fig. 3).

3.4. Anti-bovine and anti-sheep PrP antisera recognize prion epitopes

Mouse PrP residues 91–110, 144–152, and 146–159 are the targets for protective monoclonal antibodies, ICSN 35, 6H4 and ICSN 18, respectively [9,10]. Thus, we investigated whether the antisera against bovine and sheep recombinant PrPs could recognize these epitopes. Two different mouse PrP peptides, moPrP90–109 and moPrP131–154, were synthesized and subjected to a more sensitive ELISA with each concentrated (20×) antiserum of the four to five immunized mice of each group because the conventional ELISA described above was less sensitive for detecting the specific signals. This sensitive ELISA resulted in higher backgrounds from non-immunized sera (Fig. 4). However, these two peptides were recognized with the anti-bovine and the anti-sheep PrP sera, showing higher OD_{450} values compared to non-immunized sera (Fig. 4).

Mouse PrP residues 220–231 form target epitopes for PrP-specific Fab fragments, termed R1 and R2, both of which are capable of clearing PrP^{Sc} from prion-infected N2a neuroblastoma cells [12]. We similarly performed the ELISA with a synthetic moPrP219–231 peptide (Fig. 3). Higher specific absorbance could be detected in the anti-bovine and anti-sheep sera, compared to non-immunized sera. However, we could not detect any therapeutic effects of these antisera using prion infected N2a cells (data not shown). This is probably due to very low titers of the antibodies against the peptide in these antisera, as the specific signals were undetectable by conventional ELISA.

4. Discussion

In the present study, we showed that BALB/c mice immunized with bovine recombinant PrP exhibited slightly but significantly extended survival after peripheral infection with the mouse-adapted Fukuoka-1 prion. In contrast, we could not detect any prophylactic effects against the prion in mice immunized with mouse recombinant PrP. Instead, the disease seemed to be accelerated in most of the immunized mice. Sheep recombinant PrP had variable effectiveness against the prion infection. Five out of seven immunized mice developed the disease later than non-immunized mice. However, the disease seemed to be exacerbated in two remaining mice with incubation times as short as those of mice immunized with mouse recombinant PrP. These results indicate that immu-

nization effects of recombinant bovine, sheep, and mouse PrPs on the prion infection were different in BALB/c mice.

We showed that heterologous bovine and sheep recombinant PrPs, but not mouse PrP, were immunogenic in mice, stimulating antibody responses against the respective immunizing antigens. Interestingly, we also showed that mice immunized with bovine and sheep recombinant PrPs variably produced a considerable amount of anti-PrP autoantibodies, and that these anti-PrP autoantibodies could react with the mouse PrP epitopes, moPrP90–109, moPrP131–154, and moPrP219–231. White et al. showed that passive immunization of mice with anti-PrP antibodies, which recognize the epitopes overlapping the two former epitopes, moPrP90–109 and moPrP131–154, efficiently blocked prion infection [10]. It has been also reported that titers of anti-PrP autoantibodies, which were induced by immunization with mouse recombinant PrP, were well correlated to the onset time of disease in mice inoculated with mouse 139A prion [13]. It is therefore likely that autoantibody-mediated humoral immunity could be associated with the attenuation of the Fukuoka-1 prion in mice immunized with bovine and sheep recombinant PrPs. However, at the present time, we do not know the exact mechanism of the protective effects of bovine and sheep recombinant PrP immunization on prion infection. It was reported that a cytotoxic T cell-like clone could be isolated by immunization of PrP-null mice with a PrP-derived peptide conjugated with keyhole limpet hemocyanin [14]. This might indicate an alternative possibility that cellular immunity may be involved in protection against prion infection.

The prophylactic effects of the immunization of mice with recombinant sheep PrP on the prion infection seemed variable, compared with those of recombinant bovine PrP. Western blotting of bovine PrP^C with the anti-bovine PrP sera revealed that specific antibody responses were variable in the mice immunized with bovine recombinant PrP. Mice immunized with sheep recombinant PrP also showed variable antibody responses. The titers of anti-PrP autoantibodies were also various in amounts in the mice immunized with sheep or bovine recombinant PrP but seemed more variable in the mice with recombinant sheep PrP than in the mice with recombinant bovine PrP. One mouse immunized with recombinant sheep PrP elicited very weak autoantibody responses, showing OD_{405} values as low as those of mice immunized with recombinant mouse PrP, while the other mouse produced anti-PrP autoantibodies higher than any mice immunized with recombinant PrP. Moreover, on Western blotting, specific antibody responses seemed weaker in the mice immunized with sheep PrP than in the mice immunized with bovine PrP. The titers of anti-mouse PrP autoantibodies also seemed lower and autoantibodies against moPrP90–109 and moPrP219–231 were significantly less produced in the mice with recombinant sheep PrP than in the mice with bovine recombinant PrP. It is therefore suggested that this more variable and lower amount of anti-PrP autoantibodies may reflect variable and less effective protection from the disease in mice immunized with recombinant sheep PrP, compared with that of the mice immunized with bovine recombinant PrP. However, unfortunately, because we did not individually identify the immunized mice, we could not directly compare the autoantibody titers to length of the incubation times in mice immunized with recombinant bovine and sheep PrPs in the present study. Thus, at this point, we are unable to directly answer the question why the immunization effects of recombinant sheep PrP on the prion infection were more variable than those of recombinant bovine PrP or why the two mice immunized with sheep recombinant PrP succumbed to the disease earlier than control non-immunized mice.

The disease also seemed to be exacerbated in the mice immunized with mouse recombinant PrP. It was reported that complement components C3 and C1q mediate the initial trapping of prions in lymphoreticular tissues [15,16]. Therefore, complement components, which might be upregulated by immunization, may be associated with the slight, but not significant, exacerbation of the disease. Alternatively, certain conditions induced in the peritoneal cavity by multiple immunizations could be considered to be involved in the disease exacerbation because the prion was inoculated into the same peritoneal cavity. However, these remain to be elucidated.

It was previously shown that recombinant mouse PrP was immunogenic eliciting anti-PrP autoantibodies in CD-1 mice and could slightly retard onset of the disease in immunized mice after inoculation with a mouse-adapted 139A prion [13]. However, we detected only a very weak antibody response in BALB/c mice immunized with mouse recombinant PrP and no such prophylactic effects of the immunization on the prion infection. Polymenidou et al. also reported that recombinant mouse PrP failed to induce anti-PrP autoantibodies in C57BL/6× 129Sv mice [17]. The different genetic background of mice used in each experiment may be responsible for the different antibody responses. Gilch et al. reported successfully inducing anti-PrP autoantibodies by immunization of mice with mouse recombinant PrP [18]. In this case, the recombinant PrP was inserted by a human or hamster-derived 3F4 epitope at the corresponding region, resulting in the recombinant PrP with two different amino acids from mouse PrP [18]. Thus, the recombinant PrP might acquire heterologous PrP-like immunogenicities in part and thereby induce anti-PrP autoantibodies in mice.

Molecular mimicry between microbial and host antigens is a well-known hypothetical mechanism for triggering autoimmune diseases via production of autoantibodies and/or autoreactive T cells [19,20]. This hypothesis postulates that shared identical amino acid sequences or homologous but non-identical amino acid sequences between microbial and host antigens could be essential for the initial processes of molecular mimicry [19,20]. PrPs are highly conserved molecules among mammals, sharing marked similarities in both amino acid sequence and tertiary structure [21–23]. Bovine and sheep recombinant PrPs contain 19 and 21 amino acids different from mouse recombinant PrP, respectively, indicating that the higher immunogenicity of bovine and

sheep recombinant PrPs in mice might be attributable to these different amino acids. About half of these different amino acids in bovine and sheep PrPs are concentrated in the regions corresponding to moPrP90–109, moPrP131–154, and moPrP219–231. Bovine and sheep PrPs possess 2 and 3, 4 and 3, and 4 and 4 different amino acids in the corresponding moPrP90–109, moPrP131–154, and moPrP219–231 regions, respectively. It is therefore possible that these regions of bovine and sheep PrPs are immunogenic in mice because of the different amino acid composition, eliciting antibodies, which were not only specific to themselves but also to the corresponding mouse epitopes. In other words, heterologous bovine and sheep PrPs might mimic host mouse PrP to overcome tolerance. Taken together, our present results might open a new avenue for development of molecular mimicry-based prion vaccines.

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ORIGINAL PAPER

Immunohistochemical characterization of cell types expressing the cellular prion protein in the small intestine of cattle and mice

Kohtaro Miyazawa · Takashi Kanaya · Sachi Tanaka · Ikuro Takakura · Kouichi Watanabe · Shyuichi Ohwada · Haruki Kitazawa · Michael T. Rose · Suehiro Sakaguchi · Shigeru Katamine · Takahiro Yamaguchi · Hisashi Aso

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Abstract The gastrointestinal tract is thought to be the main site of entry for the pathological isoform of the prion protein (PrP^{Sc}). Prion diseases are believed to result from a conformational change of the cellular prion protein (PrP^{c}) to PrP^{Sc}. Therefore, PrP^{c} expression is a prerequisite for the infection and spread of the disease to the central nervous system. However, the distribution of PrP^{c} in the gut is still a matter of controversy. We therefore investigated the localization of PrP^{c} in the bovine and murine small intestine. In cattle, most PrP^{c} positive epithelial cells were detected in the duodenum, while a few positive cells were found in the jejunum. PrP^{c} was expressed in serotonin producing cells. In bovine Peyer's patches, PrP^{c} was distributed in extrafollicular areas, but not in the germinal centre of the jejunum and ileum. PrP^{c} was expressed in myeloid lineage cells such as myeloid dendritic cells and macrophages. In mice, PrP^{c} was expressed in some epithelial cells throughout the small intestine as well as in cells such as follicular dendritic cell in the germinal centre of Peyer's patches. In this study, we demonstrate that there are a number of differences in the localization of PrP^{c} between the murine and bovine small intestines.

Keywords Prion protein · Bovine small intestine · Murine small intestine · Peyer's patch · Immunohistochemistry

K. Miyazawa · T. Kanaya · S. Tanaka · I. Takakura · K. Watanabe · S. Ohwada · T. Yamaguchi · H. Aso (✉)
Laboratory of Functional Morphology,
Graduate School of Agricultural Science,
Tohoku University, 1-1 Tsutsumidori-Amamiyamachi,
Aoba-ku, Sendai 981-8555, Japan
e-mail: asosan@bios.tohoku.ac.jp

H. Kitazawa
Laboratory of Animal Products Chemistry,
Graduate School of Agricultural Science,
Tohoku University, 1-1 Tsutsumidori-Amamiyamachi,
Aoba-ku, 981-8555 Sendai, Japan

M. T. Rose
Institute of Rural Sciences, University of Wales,
Aberystwyth, Cardiganshire, SY23 3AL, UK

S. Sakaguchi · S. Katamine
Molecular Microbiology & Immunology,
Graduate School of Biomedical Science,
Nagasaki University, 1-12-4 Sakamoto,
Nagasaki, 852-8523 Japan

Introduction

The normal cellular isoform of the prion protein (PrP^{c}) is a highly conserved glycosylphosphatidylinositol (GPI)-anchored sialoglycoprotein. PrP^{c} is expressed in particular in the central nervous system (CNS) and its function is as yet unclear. However, it is widely accepted that the conversion of PrP^{c} into a detergent insoluble-, relatively protease-resistant isoform prion protein is a defining event in the pathogenesis of transmissible spongiform encephalopathies (TSEs) (Prusiner 1998). This form is called the disease-associated form (PrP^{Sc}). PrP knockout mice are resistant to scrapie infection (Bűeler et al. 1993; Manson et al. 1994). Therefore, PrP^{c} expression is thought to be a prerequisite for the infection and spread of the infectious agents to the CNS. TSEs are fatal neurodegenerative diseases that affect both humans and animals. They include Creutzfeldt–Jakob disease (CJD), Gertsmann-Sträussler-Scheinker syndrome and kuru in the human, scrapie in

sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle.

In 1997, it was reported that variant CJD in humans was most likely due to the transmission of BSE because of the consumption of BSE agents-contaminated foods (Will et al. 1996; Bruce et al. 1997; Hill et al. 1997). Both naturally and experimentally, the spread of TSEs by ingestion of the infectious agents has been described in a variety of species ranging from domestic animals to humans (Marsh et al. 1991; Foster et al. 1993; Wells et al. 1994; Will et al. 1996; Bons et al. 1999; Heggebø et al. 2000; Herzog et al. 2004). As described above, the oral route of infection is widely assumed to be important under natural conditions. Following an oral challenge of rodents with scrapie, the infectious agents first accumulate in Peyer's patches, gut associated lymphoid tissues and the ganglia of the enteric nervous system (Beekes and McBride. 2000; Gonzalez et al. 2005; Bergstrőm et al. 2005). Therefore, the gastrointestinal (GI) tract, especially the intestine, is thought to be a significant site of entry and first replication of the infectious agents.

The conformational conversion of PrP^{c} into PrP^{Sc} is a key step in the pathogenesis of TSEs by the widely accepted protein-only hypothesis (Prusiner 1982). To understand the critical process of TSE infection, it is important to characterize the cell types expressing PrP^{c} in the GI tract. In spite of the putative entry site, relatively few studies of PrP^{c}-expressing cells in the GI tract have been conducted. Those that have been carried out have been done mainly in rodents (Fournier et al. 1998, 2000; Ford et al. 2002; Marcos et al. 2004). However, these results lack consistency. In cattle, only one paper has reported the immunoreactivity of PrP^{c} in the bovine GI tract (Marcos et al. 2005b). In addition, it has been reported that PrP^{c} mRNA (Caughey et al. 1988; Brown et al. 1990), and PrP^{c} assessed by western blot analysis (Horiuchi et al. 1995), are widely detected in non-neuronal tissues. However, the cell types expressing PrP^{c} in the bovine intestine have not yet been established. The aims of this study were to reveal the distribution of PrP^{c} in the bovine small intestine (duodenum, Jejunal solitary Peyer's patches and ileal continuous Peyer's patches) and to characterize the cell types producing PrP^{c}.

Materials and methods

Animals and tissue preparations

Three Holstein calves (male, 6 weeks old), three BALB/c mice (male, 3 weeks old) and three *Ngsk* $Prnp^{0/0}$ mice (male, 3 weeks old) (Sakaguchi et al. 1996) were used in this study. All animals were clinically healthy and free of infectious disease. This study was conducted in accordance with the Guidelines for Animal Experimentation in Tohoku University. Immediately after slaughter, the duodenum, the jejunum with solitary Peyer's patches and the ileum with continuous Peyer's patches were dissected from the Holstein calves and immersed in 4% paraformaldehyde in phosphate buffered saline (PBS; PH 7.4) overnight at 4°C. The murine duodenum was placed in periodate lysine paraformaldehyde (PLP) fixative overnight at 4°C. After fixation, the tissue samples were paraffin-embedded and 2-μm-thick sections were made. In order to carry out immnohistochemistry for the CD markers, sections of the bovine intestine were snap-frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) and 5-μm-thick cryosections were made.

Primary antibodies

Two kinds of anti-PrP^{c} antibodies were used in this study: a rabbit antibody against the sequence of amino acids between 148 and 164 of bovine PrP^{c} (diluted 1/2,000, LSL, Tokyo, Japan) (Nakamura et al. 2002) for the bovine samples and a rabbit antibody against the sequence of amino acids between 1 and 50 at the N-terminus of human PrP^{c} (diluted 1/300, IBL, Gunma, Japan) for the murine samples. In addition, a number of other antibodies were used for the further identification of PrP^{c} positive cells (Table 1).

Immunohistochemistry

Paraffin sections were mounted on silane-coated slides, deparaffinized in xylene and rehydrated in a series of graded ethanol and water solutions. After this, endogenous peroxidase was blocked with 3% H_2O_2 for 5 min. Slides were washed with distilled water, placed in Target Retrieval Solution (Dako Cytomation, Carpinteria, CA) and heated in an autoclave for 5 min at 121°C as an antigen retrieval technique (Miyazawa et al. 2006a). Background blocking was performed with normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min before incubation with a specific antiserum. The sections were incubated overnight at 4°C with the anti-PrP^{c} antibody, rinsed in PBS and incubated with biotynilated goat anti-rabbit IgG (diluted 1/200; Vector Laboratories) for 40 min. Following this, the sections were treated with an ABC-PO kit (Vector Laboratories) for 1 h, visualized by 3,3′-diaminobenzidine tetra-

Table 1 Antibodies for identification of PrP^c-positive cells

Specificity	Species	Developed in	Clone #	Dilution	Products[a]
Chromogranin A	Human	Mouse	LK2H10	1/200	PROGEN
Gastrin/CCK	Human	Rabbit		1/2,000	AFFINITI
Somatostatin	Human	Rabbit		1/200	CHEMICON
Serotonin	Human	Rabbit		1/400	CHEMICON
CD3[b]	Bovine	Mouse	MM1A	1/50	VMRD
CD172a[c]	Bovine	Mouse	DH59B	1/50	VMRD

[a] Products from ROGEN Biotechnik GmbH (Heidelberg, German), AFFINITI Research Products Ltd. (Exeter, UK), CHEMICON INTERNATIONAL (Temecula, CA) and VMRD Inc. (Pullman, WA)

[b] T cell marker

[c] Myeloid cell marker including DC and macrophage

hydrochloride (DAB) and then counterstained with Mayer's hematoxylin. To test the specificity of immunostaining of bovine tissue, negative controls were run in which the primary antibody was omitted or replaced with an irrelevant rabbit IgG. In addition, adsorption controls were performed. Briefly, antisera against PrP^c (LSL) were preincubated for 14 h at 4°C with the bovine-recombinant PrP^c (amino acids 25–244 of bovine PrP^c; Chemicon) before application to the tissue sections.

Immunocytochemical restaining method

All polyclonal antibodies used in this study were developed in rabbits. To visualize the structure of intestinal tissue clear, we selected the re-staining method. After immunohistochemical detection of PrP^c, the paraffin sections were placed in a citrate buffer (0.01M; pH 7.4), heated in an autoclave for 5 min at 121°C to elute the anti-PrP^c antibodies, and incubated with various antisera against some neuroendocrine markers overnight at 4°C. The sections were rinsed in PBS, incubated with FITC conjugated goat anti-rabbit IgG (diluted 1/400; Sigma, St Louis, MO) for 1 h, and counterstained with propidium iodide (PI; Sigma). The sections were observed and photographed by confocal laser microscopy (MRC-1024; BioRad, Alfred Nobel Drive Hercules, CA). Following microscopic observation, the coverslips were removed and counterstained with Mayer's hematoxylin, and the same section was then re-observed. For cryosections, the sections were incubated with two kinds of mouse monoclonal antibodies against CD markers overnight at 4°C. The sections were rinsed in PBS, incubated with FITC conjugated goat anti-mouse IgG (diluted 1/400; Sigma) for 1 h, and counterstained with PI (Sigma). The observation of these sections was done using a similar method to that described above.

Results

Localization of PrP^c-positive cells in the bovine and murine small intestine

PrP^c–positive cells were found throughout the small intestine of calves and mice, and the staining for PrP^c on cryosections was the same as for paraffin sections. In calves, PrP^c-positive epitheliocytes were observed in the basal region of the duodenum crypts (Fig. 1d), but not in the villi (Fig. 1a–c, f, j, k). In the jejunum, PrP^c-positive epitheliocytes were seldom detected in the villous crypts and undetectable nearby in the ileum (Fig. 1h, l). Immunostained cells in Peyer's patches were detected in the dome region under the follicle-associated epithelium (FAE), but not in B cell follicles (Fig. 1e, f, i, j). These cells possessed granular PrP^c-immunoreactivity in their cytoplasm (Fig. 1g, k). Similar cells were occasionally observed in lamina propria of the duodenal villi (Fig. 1c). These immunoreactivities in the bovine small intestine were not detected in negative and absorption controls.

In mice, PrP^c-positive epitheliocytes appeared randomly in the epithelium of the villi and crypts throughout the small intestine (Fig. 2b, c). These epithelial cells might be identified as neuroendocrine cells on the basis of their morphology. Some of these cells had the typical morphology of the intestinal open endocrine cells with long apical processes reaching the lumen (Fig. 2c). PrP^c-positive cells were also observed in the epithelia of the basal regions of the crypts (Fig. 2b). These cells had the morphology of the closed endocrine cell type. PrP^c was detected in follicular dendritic cell (FDC)-like cells within the B cell follicle (Fig. 2a, e), but not in the dome region under the FAE of the Peyer's patches (Fig. 2d). In contrast, there was no PrP^c-immunoreactivity in *Ngsk* $Prnp^{0/0}$ mice (Fig. 2f–j). These data suggest that there are differences in the pattern of distribution as well as in

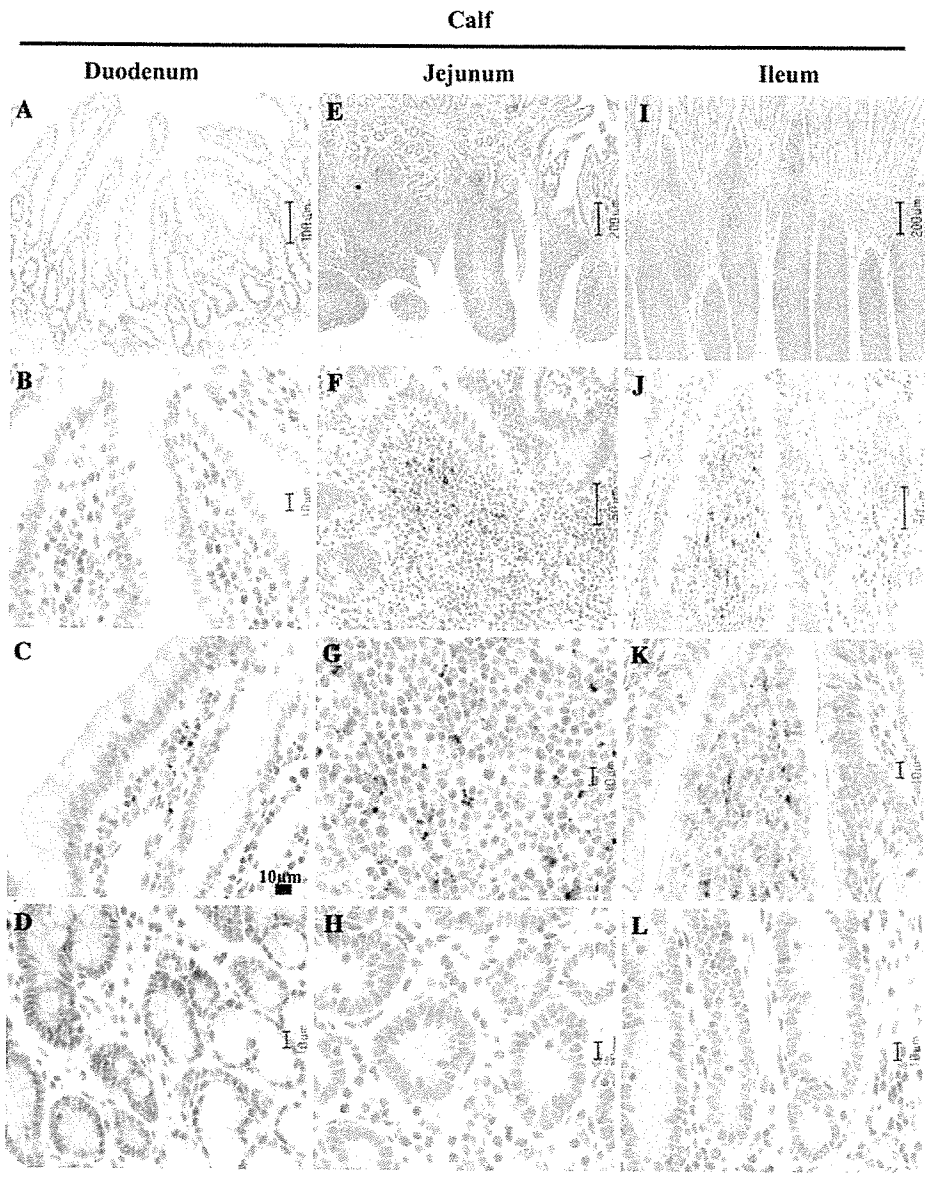

Fig. 1 Localization of PrP^c-positive cells in bovine small intestine. Immunohistochemical micrographs show bovine duodenum (**a–d**), jejunum (**e–h**) and ileum (**i–l**). In the bovine duodenum, PrP^c was clearly observed in some epithelial cells close to the crypt and the lamina propria (**c**, **d**), but very weakly in the jejunal solitary and the ileal continuous Peyer's patches (**h**, **l**). Immunopositive granules were detected in the dome region of bovine jejunal and ileal Peyer's patches (**g**, **k**)

the nature of the cells expressing PrP^c in the Peyer's patches of calves and mice.

Identification of cell types expressing PrP^c in the bovine small intestine

Figure 1d shows that PrP^c-positive epithelial cells in calves looked like neuroendocrine cells on the basis of their morphology. In order to determine the nature of the epitheliocytes displaying PrP^c immunoreactivity, double immunostaining was performed (Fig. 3). In the bovine duodenum, all PrP^c-positive cells expressed chromogranin A, indicating that they were neuroendocrine cells (arrows in Fig. 3a, b). In addition, all PrP^c-positive cells expressed 5HT (arrows in Fig. 3c, d); however, not all the 5HT-positive cells also expressed PrP^c (arrowheads). Any other neuroendocrine markers, such as gastrin and somatostatin, were not expressed in PrP^c-immunolabeled epithelial cells (Fig. 3e–h).

In bovine jejunal solitary and ileal continuous Peyer's patches, PrP^c-positive cells were detected in the dome region under the FAE. These were considered to be lymphoid cells, because of their distribution (Fig. 1f, i). Therefore, we performed a dual immunostaining for PrP^c and CD markers in order to identify the lymphoid cell type (Fig. 4). As for the immunohistochemistry results, PrP^c-positive cells of the dome region were myeloid-lineage cells with a CD172a marker (arrows in Fig. 4a, b, e, f), but not T cells with

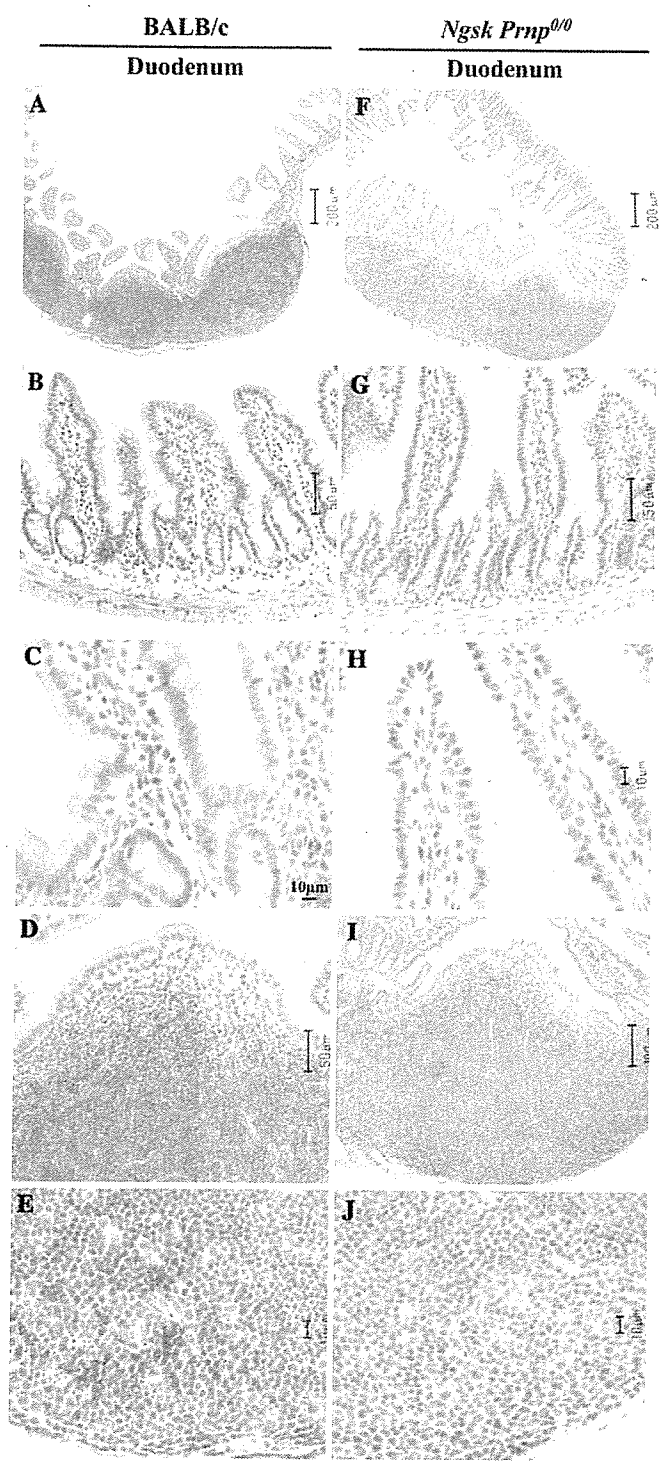

Fig. 2 Localization of PrP^c-positive cells in murine small intestine. Immunohistochemical micrographs show duodenum of BALB/c mice (**a–e**) and prion protein knockout mice (*Ngsk Prnp*$^{0/0}$) (**f–j**). The higher magnification photographs showed that some epithelial cells were clearly stained with PrP^c nearby the crypt and in the crypt on the side of villi (**b**, **c**), but not of the FAE (**d**). PrP^c was detected within the germinal centres of murine Peyer's patches (**a**, **e**) in contrast to bovine Peyer's patches (Fig. 1). No PrP^c-immunoreactivity was observed in *Ngsk Prnp*$^{0/0}$ mice (**f–j**)

CD3 (arrowheads in Fig. 4c, d, g, h). In addition, these myeloid cells with PrP^c penetrated through the FAE (Fig. 4e, f). These data indicate that PrP^c is strongly expressed by myeloid origin cells such as dendritic cells (DCs) and macrophages in bovine Peyer's patches.

Discussion

Recently, it has been reported that PrP^c is expressed in bovine duodenal epithelium (Marcos et al. 2005b), and this conclusion is consistent with our data. In our

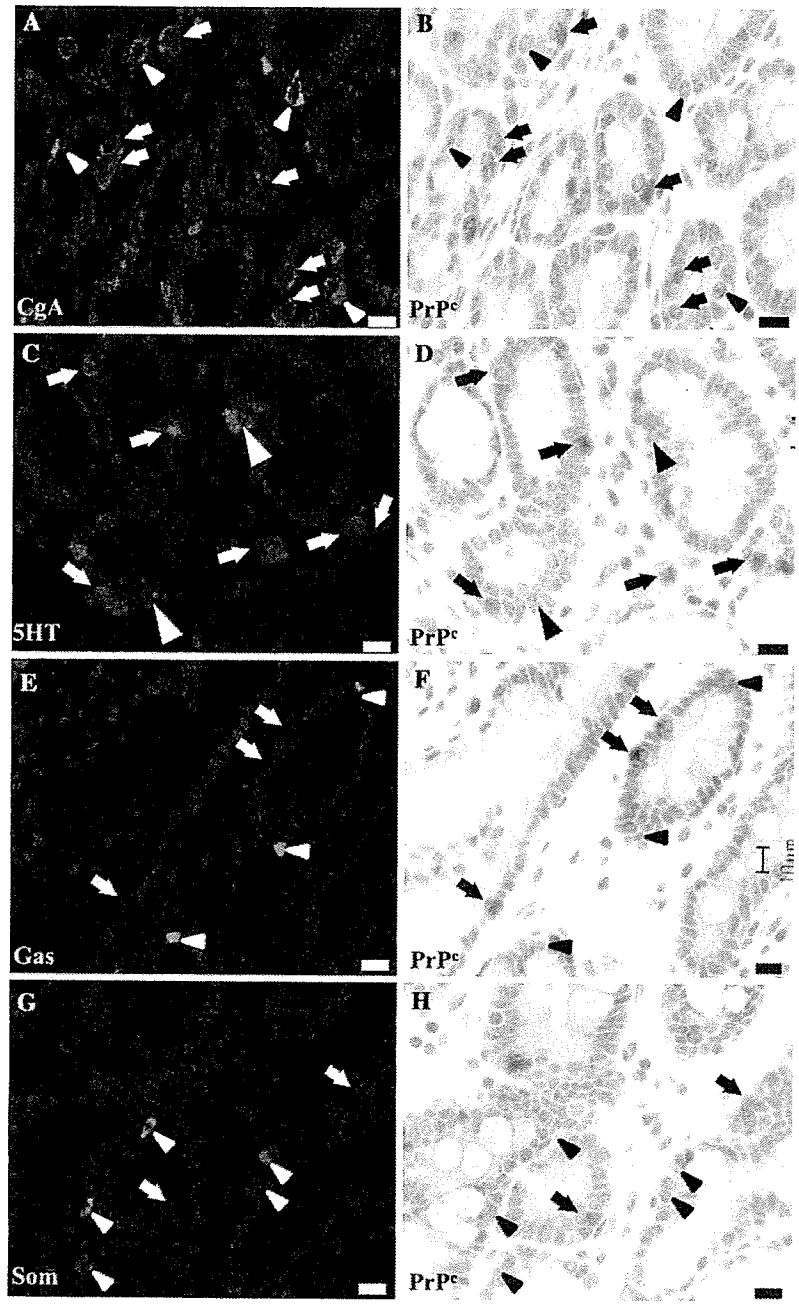

Fig. 3 Dual immunostaining for PrP^c and various neuroendocrine markers in the bovine duodenum. The paraffin sections of the bovine duodenum were performed to the immunohistochemical restaining, using anti-PrP^c antibody and four kinds of antibodies against a neuroendocrine markers. The same section is shown in the *left* and *right* photograph in each *row*. The photographs in the *left* column showed the immunostaining for chromogranin A (*CgA*, **a**), serotonin (*5HT*, **c**), gastrin/CCK (*Gas*, **e**) and somatostatin (*Som*, **g**). The photographs in the *right* column showed the immunostaining for PrP^c (**b**, **d**, **f**, **h**). *Arrows* and *arrowheads* pointed to PrP^c-positive cells and the cells only with neuroendocrine markers, respectively. All PrP^c-immunoreactive epithelial cells expressed CgA (*arrows* in **a**, **b**) and 5HT (*arrows* in **c**, **d**). However, there were 5HT-immunoreactive epithelial cells without PrP^c. *Green* is the subset of neuroendocrine markers and *red* are the nuclei of all cells. *Bars* 10 μm

study, the majority of PrP^c-positive cells were observed in the bovine duodenal epithelium. The number of PrP^c-positive epithelial cells was decreased close to jejunal solitary Peyer's patches, but not in ileal continuous Peyer's patches. In addition, all PrP^c-positive epithelial cells were identified as serotonin (5HT) producing cells. The previous reports of studies on rodents (Ford et al. 2002; Marcos et al. 2004) and monkeys (Marcos et al. 2005a) show that PrP^c is co-localized with 5HT-producing cells, in common with our data. We also detected PrP^c-positive epithelial cells in the murine intestine, which had the typical morphology of neuroendocrine cells. Ford et al. (2002) have also reported that PrP^c-positive cells in the murine mucosal wall of the gut are endocrine cells producing 5HT. However, it has been reported that PrP^c is detected in cells producing not only serotonin but also somatostatin and gastrin in the rat (Marcos et al. 2004) and monkey small intestine (Marcos et al. 2005a). On the other hand, it has been reported that

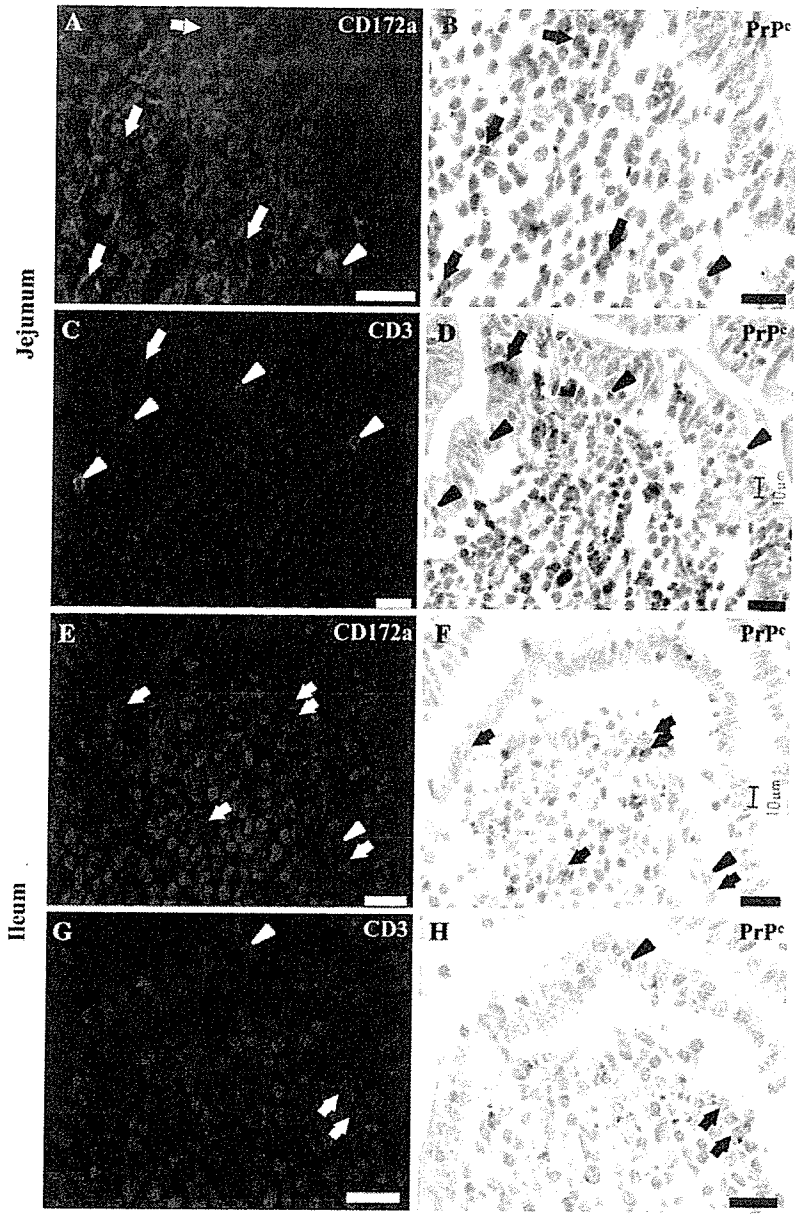

Fig. 4 Identification of PrP^c-positive cells in bovine small intestine. The cryosections of the dome region in bovine jejunal and ileal Peyer's patches were performed to the immunohistochemical re-staining, using anti-PrP^c antibody and anti-CD markers. The same section is shown in the *left* and *right* photograph in each *row*. *Arrows* and *arrowheads* point to PrP^c-positive cells and the cells only with CD markers, respectively. In jejunal solitary and ileal continuous Peyer's patch, PrP^c-immunoreactivity was observed in a subset of CD172a-positive cells, which were myeloid lineage cells including macrophages and dendritic cells (*arrows* in **a**, **b**, **e**, **f**). CD3-positive cells (T cells) did not express PrP^c (*arrowheads* in **c**, **d**, **g**, **h**). *Green* is the subset of CD markers and *red* are the nuclei of all cells. *Bars* 20 μm

the basic helix–loop–helix transcription factor neurogenin 3 (ngn3) is required for endocrine cell fate specification in multipotent intestinal progenitor cells (Jenny et al. 2002). Although ngn3 was not directly related to the PrP^c expression, unknown factors might control the production of PrP^c and 5HT.

There is no report that 5HT producing neuroendocrine cells may be closely related to TSE infection. Iwanaga et al. (1994) have reported a topographical relationship between 5HT producing cells and nerves that the nerve fibres containing vasoactive intestinal polypeptide are observed in close proximity to 5HT immunoreactive cells. We propose the hypothesis that PrP^{Sc} by oral infection may contact with PrP^c of 5HT producing cells in lumen and convert their PrP^c into the abnormal PrP^{Sc}, and then these converted PrP^{Sc} may be transferred to the proximal nerve fibres.

In murine Peyer's patches, PrP^c-immunoreactivity could not be detected in the FAE, including M cells, in common with a previous report (Ford et al. 2002). The latter authors also reported that weakly immunopositive granules were occasionally observed within the apical region of enterocytes. PrP^c was also expressed in FDC-like cells in follicle in mice. In bovine Peyer's patches, PrP^c-positive epithelial cells could not be

detected in the FAE, and PrP^c was detected in CD172a-positive cells in the dome region of Peyer's patches. It has been reported that CD172a is a myeloid lineage marker (Herrmann et al. 2003) and is expressed in bovine myeloid DCs (Miyazawa et al. 2006b). Our results are supported by the fact that myeloid DCs in human and Langerhans cells in mice strongly express PrP^c (Burthem et al. 2001; Sugaya et al. 2002).

We were not able to detect PrP^c-positive cells in bovine jejunal and ileal follicle tissues using an antibody against the 148–164 amino acid sequence of PrP^c (Fig. 1e, i). Thielen et al. (2001a) have reported that PrP^c is expressed in bovine FDCs of lymph node and tonsil, but not of the germinal centre, using SAF32 and SAF34 antibodies binding the 79–92 amino acid sequences located within the octorepeat region of PrP^c. These authors suggest two reasons why these antibodies do not react with PrP^c expressed in FDCs: (1) the 79–92 amino acid sequences of PrP^c might be inaccessible in the germinal centre or (2) the PrP^c synthesized in the germinal centre might undergo several post-translational modifications, e.g., pattern of glycosylation, folding and hydrolysis of antigenic sites.

It is interesting that the myeloid lineage cells express PrP^c in bovine Peyer's patches because myeloid DCs and macrophages have a high ability to take up and present antigens. In particular, the susceptibility to prion infection following oral challenge is thought to correlate with the number of Peyer's patches (Prinz et al. 2003). It has been shown that DCs penetrate through the gut epithelium, extend their dendrites outside the epithelium and directly sample bacteria (Rescigno et al. 2001), and that in vitro infectious agents are transported by M cells using the Caco-2 cell line (Heppner et al. 2001). We also confirmed CD172a-positive cells with PrP^c creeping between epithelial cells in the FAE (Fig. 4e, f). In addition, Huang et al. (2002) have shown that migrating intestinal myeloid DCs transport PrP^{Sc} from the gut.

PrP^{Sc} accumulation and its infectivity are easily detectable in spleen, tonsil and other lymphoid tissues during scrapie in sheep, hamsters and mice (Andreoletti et al. 2000; Schreuder et al. 1998). In addition, PrP^{Sc} accumulation is found in lymphoid tissues in the case of experimental transmission of BSE to sheep (Foster et al. 2001) and transgenic mice with expression of bovine PrP^c (Asano et al. 2006), and in the case of vCJD (Wadsworth et al. 2001). In most cases, PrP^{Sc} accumulation mainly occurs in FDCs before spreading to the nervous system (van Keulen et al. 1996; Kitamoto et al. 1991; Hill et al. 1999; Sigurdson et al. 2002). These data are in sharp contrast to the evidence that was observed during BSE in cattle. Previous reports indicated that the infectivity was found only in the central and peripheral nervous system, but not in lymphoid tissues (Buschmann and Groschup 2005), and that no PrP^{Sc} accumulation was observed in spleen of BSE-infected cattle (Somerville et al. 1997). In addition, it has been reported that the infectivity is found in the terminal ileum of cattle experimentally inoculated with end-stage clinical BSE (Wells et al. 1994), but not in that of clinically affected natural BSE cases in cattle (Terry et al. 2003). Although FDCs in sheep, hamster and mice seem to express high levels of PrP^c (McBride et al. 2002; Brown et al. 1999; Thielen et al.2001b; Bencsik et al. 2001), FDCs of bovine Peyer's patches may not express PrP^c.

Race et al. (2000) reported that PrP expression in peripheral nerves was sufficient for successful infection of the brain, and that peripheral expression of heterologous PrP completely protected the delivery of PrP^{Sc} to the brain. In addition, FDCs-deficient mice delayed the neuroinvasion and reduced the disease susceptibility (Mabbott et al. 2000, 2003; Montrasio et al. 2000), and wild-type mice had incubation time $\approx$50 days less than mutant mice, which were deficient in the functions of immune system (Schlomchik et al. 2001). These data indicate that an intact immune system including FDCs may increase agent uptake and delivery. However, it remains to be determined that how and where the infectious agent enters the GI tract in cattle, and further, how it replicates and is transported to the CNS. We speculate that myeloid lineage cells expressing PrP^c might be fundamentally involved in the propagation and replication of the infectious agents.

In conclusion, we summarized results in the text and illustrated in the figures for Table 2. We have shown that PrP^c is expressed in some but not all serotonin (5HT) producing cells in bovine duodenum, and that myeloid lineage cells such as myeloid DCs and macrophages are immunoreactive for PrP^c in bovine Peyer's patches. There is quite a difference in the distribution of PrP^c in the follicle of Peyer's patches between cattle and mice. It has been reported that PrP^c in the CNS is involved in the survival of Purkinje cells (Sakaguchi et al. 1996). However, the functional significance of the PrP^c only in the serotonin producing cells is unclear at present. Further studies, possibly using bovine small intestinal epitheliocytes in vitro are needed to understand the function of PrP^c in bovine small intestinal epitheliocytes.

Table 2 Distribution of PrP^c in bovine and murine small intestine

Region	Duodenum	Jejunum	Ileum
Bovine			
Villous epithelium	5HT-producing cells	5HT-producing cells	ND
FAE	–[a]	ND	ND
Dome region	–	$CD172a^+$ cells	$CD172a^+$ cells
Follicle	–	ND	ND
Murine			
Villous epithelium	Enteroendocrine cells	Enteroendocrine cells	Enteroendocrine cells
FAE	ND	ND	ND
Dome region	ND	ND	ND
Follicle	FDC-like cells	FDC-like cells	FDC-like cells

This table summarizes the results which are reported in the text and illustrated in the figures

ND not detectable, *FAE* follicle associated epithelium

[a] Bovine duodenum has no Peyer's patches

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Surface Plasmon Resonance Analysis for the Screening of Anti-prion Compounds

Satoshi KAWATAKE,[a] Yuki NISHIMURA,[a] Suehiro SAKAGUCHI,[b] Toru IWAKI,[c] and Katsumi DOH-URA*,[a]

[a] *Department of Prion Research, Tohoku University; Sendai 980–8575, Japan:* [b] *Department of Molecular Microbiology and Immunology, Nagasaki University; Nagasaki 852–8523, Japan: and* [c] *Department of Neuropathology, Neurological Institute, Kyushu University; Fukuoka 812–8582, Japan.*

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The interaction of anti-prion compounds and amyloid binding dyes with a carboxy-terminal domain of prion protein (PrP121—231) was examined using surface plasmon resonance (SPR) and compared with inhibition activities of abnormal PrP formation in scrapie-infected cells. Most examined compounds had affinities for PrP121—231: antimalarials had low affinities, whereas Congo red, phthalocyanine and thioflavin S had high affinities. The SPR binding response correlated with the inhibition activity of abnormal PrP formation. Several drugs were screened using SPR to verify the findings: propranolol was identified as a new anti-prion compound. This fact indicates that drug screenings by this assay are useful.

Key words anti-prion compound; surface plasmon resonance; scrapie-infected cell; screening; recombinant prion protein

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheincker syndrome in humans, and scrapie, bovine spongiform encephalopathy and chronic wasting disease in animals. These disorders are characterized by accumulation in the brain of an abnormal isoform of prion protein (PrP), which includes a high beta-sheet content and is resistant to digestion with proteinase K.[1] Recent outbreaks of variant Creutzfeldt–Jakob disease[2] and iatrogenic Creutzfeldt–Jakob disease through use of cadaveric growth hormone or dura grafts[3] in younger people have necessitated the development of suitable therapies. Compounds such as antimalarials and amyloid binding dyes are known to possess anti-prion activity *in vitro* or *in vivo*.[4—14] Among them, Congo red and quinacrine are known to bind directly to PrP and thereby strongly inhibit proteinase K-resistant PrP (PrPres) formation.[15,16] However, it remains unclear whether or not other anti-prion compounds and amyloid binding dyes interact directly with PrP. This study analyzed interactions of some previously reported anti-prion compounds[4,7,11,17,18] and popularly used amyloid binding dyes with recombinant PrP using surface plasmon resonance (SPR). In addition, we evaluated whether SPR assay is useful as a screening tool for anti-prion compounds.

MATERIALS AND METHODS

Compounds Compounds used in the study (Fig. 1) were obtained from Sigma Aldrich Corp. (quinacrine dihydrochloride (QC, MW: 400.0), quinine hydrochloride (QN, MW: 324.4), thioflavin T (ThT, MW: 283.4, dye content 65%), thioflavin S (ThS, MW: undetermined), propranolol (MW: 295.8), promethazine hydrochloride (MW: 284.4), carbamazepine (MW: 236.3) and theophylline (MW: 180.2)), Aldrich (chloroquine diphosphate (CQ, MW: 319.9), and Congo red (CR, MW: 696.7, dye content 97%)), ICN (phthalocyanine tetrasulfonate (PcTS, MW: 922.7)), Wako Pure Chemical Industries Ltd. (Tokyo, Japan) (tetracycline hydrochloride (TC, MW: 444.4), diazepam (MW: 284.7), folic acid (MW: 441.4) and phenytoin (MW: 252.3)) or Nacalai Tesque (Tokyo, Japan) (testosterone (MW: 288.4)). All compounds were prepared as 20 mM stock solutions in water or dimethyl sulfoxide.

SPR Analysis The SPR analysis was performed using an

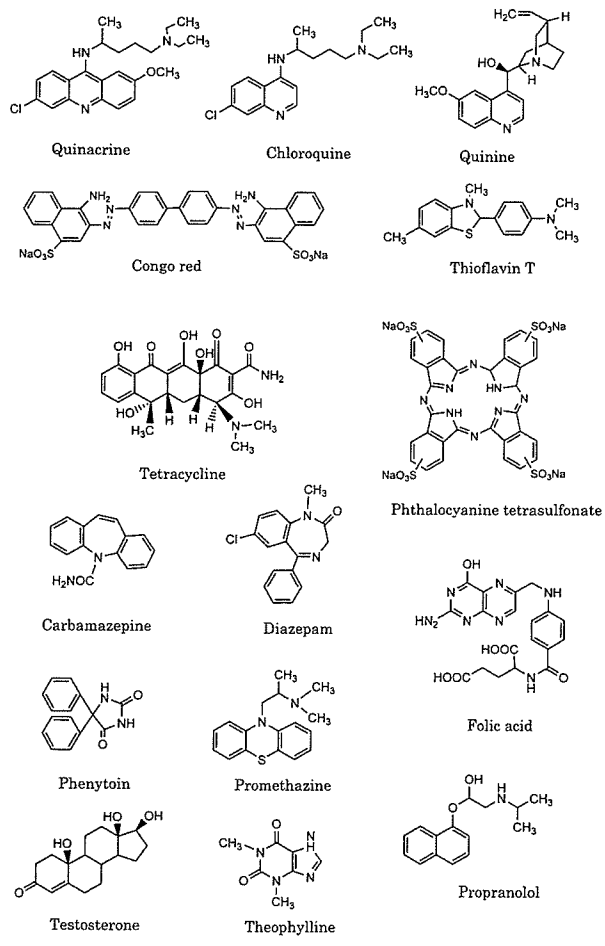

Fig. 1. Structures of Compounds or Drugs Used in the Study

* To whom correspondence should be addressed. e-mail: doh-ura@mail.tains.tohoku.ac.jp

optical biosensor (Biacore AB, Uppsala, Sweden) equipped with a CM5 sensor chip. Recombinant mouse PrP was prepared as described previously[19,20] and immobilized on a biosensor chip at a density of *ca.* 3000 resonance units (RU) using amine coupling.[21] Test compounds were diluted to 100 μM with running buffer (70 mM NaCl, 53 mM Na_2HPO_4, 12.5 mM KH_2PO_4, pH 7.4) and contained 0.5% DMSO. After they were confirmed to be in solution without precipitation or aggregation, they were injected over the PrP flow cell and the reference for either 60 s at a flow rate of 20 μl/min (low-affinity compounds) or 90 s at a flow rate of 30 μl/min (high-affinity compounds). The dissociation phase was monitored for 60 s (low-affinity compounds) or 270 s (high-affinity compounds). The flow cell was washed with 10 mM NaOH or 0.01% Triton X-100 for 30 s between each sample injection. Buffer blanks for double referencing were injected before sample analyses.[22]

The full-length recombinant of mouse PrP (residues 23—231) was used initially in the experiment, but it was easily degraded during SPR analysis in the amino-terminal portions attributable to an unidentified mechanism. For that reason, the carboxy-terminal polypeptide (residues 121—231; PrP121—231), which represents the only autonomous folding unit of PrP with a defined three-dimensional structure,[19,23,24] was used in this study.

Every PrP-immobilized biosensor chip used in the study was confirmed to respond almost the same and was standardized by the measurement of QC before its use for sample analyses.

Data Analysis The binding response, which is an index for estimating the interaction of a compound with molecules sited on a biosensor chip, is obtained from the equilibrium response (R_{eq}) value or the maximum response value in the sensorgram divided by the molecular weight.[25] In this study, the binding response of a compound was standardized by calibrating with QC, whose binding response was designated as 100 RU/Da. For low-affinity compounds, the dissociation constant (K_D) based on the R_{eq} state was calculated from data at doses ranging from 10 μM to 1 mM by either steady-state analysis using BIAevaluation software (ver. 3.0; Biacore AB) or Scatchard plot analysis. On the other hand, the K_D for high-affinity compound CR or PcTS was deduced after the data were fit to a binding model assuming a bivalent analyte in BIAevaluation software. The fitting was performed in such a way that the χ^2 value representing the statistical closeness of curve-fitting became the lowest. It was recommended ideally to be below 10.

Statistical linear correlation was evaluated using Pearson's correlation coefficient; Fisher's r to z method was used to calculate the p values. Simple linear regression analysis was also performed.

Anti-prion Activity Assay Anti-prion activity of a compound was assayed by measuring its 50% inhibition doses (IC_{50}) for PrPres formation in scrapie-infected neuroblastoma (ScNB) cells as described in previous reports.[7,11,12] Briefly, compounds were added at designated concentrations to the medium when cells were passed at 10% confluency. Cells were allowed to grow to confluence and lysed with lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS). Lysates were digested with 10 μg/ml proteinase K for 30 min and centrifuged at 100000×g for 30 min at 4 °C. The pellets were resuspended in sample loading buffer and boiled. Samples were separated using electrophoresis on a 15% Tris-glycine-SDS-polyacrylamide gel and electroblotted. PrPres was detected using an antibody SAF83 (1 : 5000; SPI-Bio, France), followed by an alkaline phosphatase-conjugated secondary antibody. Immunoreactive signals were visualized using CDP-Star detection reagent (Amersham Biosciences Corp., U.S.A.) and were analyzed densitometrically. Three independent assays were performed in each experiment.

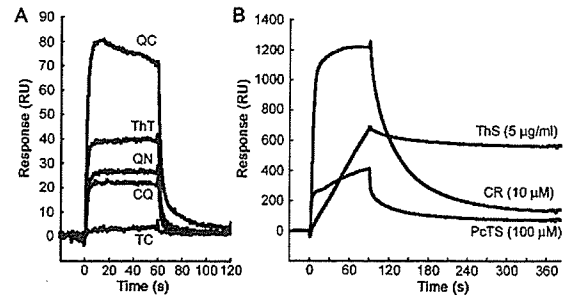

Fig. 2. Interactions of Anti-prion Compounds with PrP121—231

(A) Sensorgrams of the low-affinity compounds quinacrine (QC), chloroquine (CQ), quinine (QN), thioflavin T (ThT) and tetracycline (TC), all at 100 μM. (B) Sensorgrams of the high-affinity compounds Congo red (CR, 10 μM), phthalocyanine tetrasulfonate (PcTS, 100 μM) and thioflavin S (ThS, 5 μg/ml).

RESULTS

Interaction of Anti-prion Compounds with PrP The SPR sensorgrams of ThT and antimalarials such as QC, QN and CQ (each at 100 μM) demonstrated weak signal responses of less than 100 RU (Fig. 2A). The responses of these compounds reached equilibrium (R_{eq}) within a few seconds and returned to the baseline very rapidly after dissociation. These sensorgrams were typical for low-affinity interactions: TC showed almost no response. On the other hand, all sensorgrams of high-affinity compounds, such as CR, PcTS and ThS, showed much stronger responses and individual characteristic curves that differed from those of the low-affinity compounds (Fig. 2B). The CR (10 μM) showed the strongest signal, which was greater than 1200 RU: this decreased very slowly in the dissociation phase. The signal responses for PcTS (100 μM) and ThS (5 μg/ml) showed that neither reached the R_{eq} state within the association phase or returned to the baseline within the dissociation phase. In particular, ThS was only slightly dissociated and remained bound. This sensorgram resembled the sensorgram of biquinoline, an effective inhibitor of PrPres formation in ScNB cells (IC_{50}=3 nM).[11]

K_D Determination The dose response curve for QC appeared to be monophasic and to reach a saturation level at higher concentrations; its dissociation constant (K_D) value was calculated as 1.1 mM or 0.9 mM using steady-state analysis or Scatchard plot analysis, respectively (Figs. 3A—C). Vogtherr *et al.*[16] reported the dissociation constant (K_D= 4.6 mM) of the complex of QC and human PrP 121—230 analyzed by nuclear magnetic resonance (NMR) spectroscopy. This value was almost comparable to the K_D value obtained in this study, indicating that the method used in this study was relevant. The other two low-affinity compounds, QN and

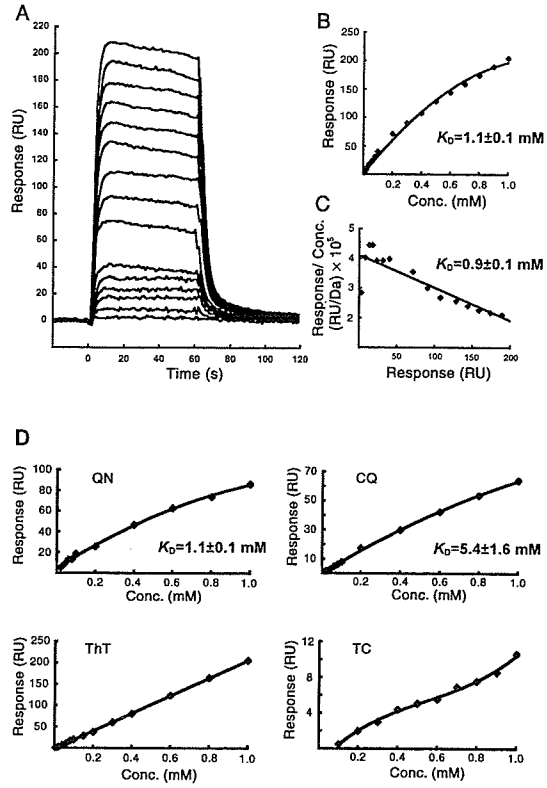

Fig. 3. Kinetic Analyses of Low Affinity Compounds

(A) Sensorgrams, (B) dose response curve and (C) scatchard plot of QC. (D) Dose response curves of QN, CQ, ThT, and TC.

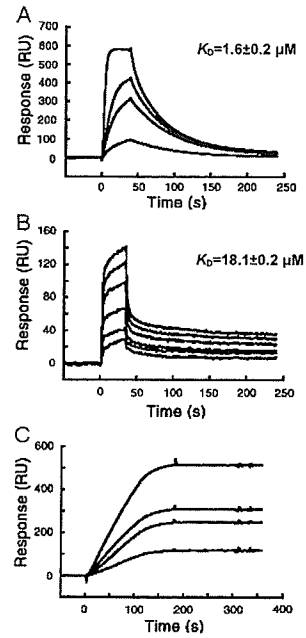

Fig. 4. Kinetic Analyses of High Affinity Compounds

(A) Sensorgrams of CR at concentrations of 1, 2, 3.3 and 5 μM, and its K_D value. (B) Sensorgrams of PcTS at concentrations of 1, 5, 10, 50, 75 and 100 μM, and its K_D value. (C) Sensorgrams of ThS at concentrations of 1, 2, 3 and 5 μg/ml, and its K_D value could not be calculated because of its undetermined structure and molecular weight.

CQ, respectively showed a similar monophasic pattern in dose response curves, yielding K_D of 1.1 mM and 5.4 mM (Fig. 3D). These K_D values, however, were of rough estimation and might be a little underscored due to lack of the data at concentrations of more than 1 mM. Unstable solubility of the compounds at such high concentrations hindered further analyses.

On the other hand, ThT gave a linear dose–response curve within a concentration of up to 1 mM and TC showed a biphasic pattern (Fig. 3D). Therefore, the saturation levels and K_D values of these compounds could not be determined, indicating that these compounds have a very low or no affinity with PrP121—231. Of them, TC is known to revert abnormal physicochemical properties of PrPres *in vitro*,[18] and interaction between TC and human PrP 106—126 peptides is revealed by NMR analysis.[26] Their data appear to be inconsistent with the data in this study. However, this discrepancy might be attributable to the lack of a TC binding site in the PrP121—231 used in our study.

Each sensorgram of high affinity compounds showed a very slow dissociation phase and was individually characteristic (Fig. 4). The structural and stoichiometric binding details of the compounds with PrP121—231 have not yet been established, but CR or PcTS is a symmetrical molecule and either half of the molecule has anti-prion activity (Doh-ura K, unpublished data). Consequently, the K_D value for the compound was deduced after the data were fit to a binding model assuming a bivalent analyte. The K_D of CR was calculated to be 1.6 μM from the sensorgrams of 1, 2, 3.3 and 5 μM (χ^2=20.9±2.1) (Fig. 4A). The K_D of PcTS was calculated as 18.1 μM from the sensorgrams of 1, 5, 10, 50, 75 and 100 μM (χ^2=28.1±2.9) (Fig. 4B). The K_D of ThS was incalculable to an exact degree because it is presumed to be a mixture of compounds formed by methylation and sulfonation of primulin; their structures and molecular weights have not been determined.

Comparison between PrP Affinity and Anti-prion Activity The IC_{50} value for the inhibition of PrPres formation in ScNB cells, either previously reported or examined in this study, was used as an anti-prion activity in this study. It was compared with the K_D or with the binding response. The latter, an index for estimating the interaction, was obtained from the R_{eq} value or the maximum response value at a concentration of 1 mM divided by the molecular weight (Table 1).

From data of all compounds except ThT, TC and ThS, statistical analyses demonstrated a significant linear correlation between the reciprocal of binding response and the IC_{50} (r=0.985, p=0.0005) (Fig. 5). This relation appeared to be also observed in TC, but not in ThT showing the next highest binding response to QC but no inhibition of PrPres formation within a non-toxic dose range. However, ThT demonstrated cell-toxicity at such a low dose as 0.05 μM.

For ThS, assuming that its minimum molecular weight deduced from presumable structures was 520 Da, its binding response was estimated to be 5.03 RU/Da; the IC_{50} was estimated to be about 2 μM, corresponding to about 1 μg/ml. However, these values seem to be underestimates because some constituents of ThS might interact with PrP121—231 or have inhibitory activity for PrPres formation. Therefore, active constituents of ThS might be expected to inhibit PrPres formation in ScNB cells at a submicromolar dose, similar to the other high-affinity compounds.

Screening by SPR Findings suggested that a compound

Table 1. Binding Response, Dissociation Constant (K_D) and 50% PrPres Inhibition Dose in ScNB Cells (IC_{50})

Compound	Binding response[a] (RU/Da)	K_D[b] (mM)	IC_{50}[c] (μM)
Low-affinity			
Quinacrine (QC)	0.25±0.00	1.1±0.1 (0.9±0.1)	0.3 (7)
Quinine (QN)	0.05±0.00	1.1±0.1 (1.4±0.1)	6.0 (11)
Chloroquine (CQ)	0.07±0.01	5.4±1.6 (3.5±0.8)	4.0 (7)
Thioflavin T (ThT)	0.16±0.01	n.d.[d] (n.d.[d])	No effect[f]
Tetracycline (TC)	0.01±0.00	n.d.[d] (n.d.[d])	No effect[g]
High-affinity			
Congo red (CR)	8.74±0.64	$1.6\pm0.2\times10^{-3}$	1.5×10^{-2} (4)
Phthalocyanine tetrasulfonate (PcTS)	1.82±0.06	$18.1\pm0.2\times10^{-3}$	0.5 (17)
Thioflavin S (ThS)	n.d.[e]	n.d.[e]	*ca.* 1 μg/ml

a) Binding response value was calculated from the R_{eq} value divided by the molecular weight for QC, QN, CQ and CR, or from the response value at a concentration of 1 mM divided by the molecular weight for ThT, TC and PcTS. *b*) K_D values were determined by steady state analysis for the low-affinity compounds or by bivalent analyte model analysis for CR and PcTS. K_D values from Scatchard plot analyses are shown in parentheses. *c*) IC_{50} values reported in the literature (reference shown in parentheses) or examined in this study. *d*) n.d.: not determined because a saturation level could not be estimated. *e*) n.d.: not determined because its structure and molecular weight were undetermined. *f*) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 0.05 μM. *g*) Inhibition of PrPres formation was not observed up to a minimal toxic dose of 5.0 μM.

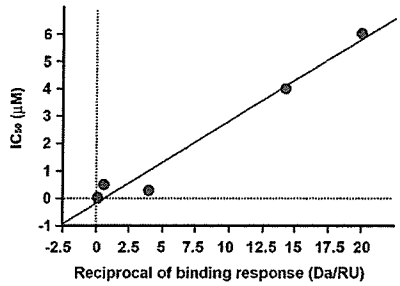

Fig. 5. Correlation between the Reciprocal of Binding Response and the IC_{50}

The data were from five compounds in which both binding response and IC_{50} were determined. Correlation showed a slope of 0.298, an intercept of −0.156 and a correlation coefficient of 0.971 (p=0.002) by simple linear regression analysis.

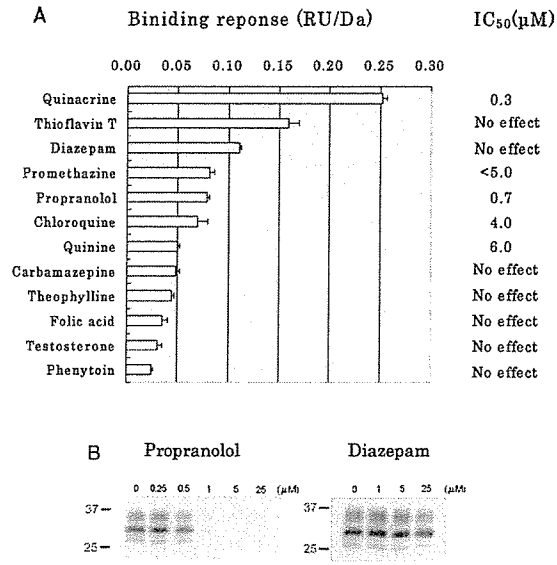

Fig. 6. Screening of Anti-prion Candidates Using the SPR Assay

(A) Binding response of each sample at 100 μM, and its IC_{50} of PrPres formation inhibition in ScNB cells within a non-toxic dose range. (B) Inhibition analyses of PrPres formation in ScNB cells grown in the presence with propranolol or diazepam. Molecular sizes in kDa are shown at the left of each panel.

capable of interacting with PrP121—231 might have a potency of inhibiting PrPres formation in ScNB cells. To verify this inference, several drugs were examined for either their binding response using the SPR method or their IC_{50} in ScNB cells. Eight clinically utilized drugs—carbamazepine, diazepam, folic acid, phenytoin, promethazine, propranolol, testosterone, and theophylline—all of which are low molecular weight compounds capable of crossing the blood brain barrier and share a partial structure similarity with the anti-prion compounds already reported, were examined and compared with the four anti-prion compounds (QC, QN, CQ, and ThT) (Fig. 6A).

Diazepam, promethazine and propranolol showed a higher binding response value than QN, which was the lowest binding response compound among the effective anti-prion compounds examined in this study. Among these, promethazine or propranolol inhibited PrPres formation in ScNB cells (propranolol: IC_{50}=0.7 μM; promethazine: IC_{50}<5.0 μM). Promethazine has already been reported to have anti-prion activity in ScNB cells,[8] whereas propranolol is a novel compound that inhibits PrPres formation in ScNB cells. Diazepam apparently did not inhibit PrPres formation within a non-toxic dose range up to 25 μM (Fig. 6B). Inhibitory activities against PrPres formation in ScNB cells were not observed for other drugs that had lower binding response values than QN.

DISCUSSION

We demonstrated that most anti-prion compounds examined in this study interacted with PrP121—231. The binding response of the compounds correlated with the IC_{50} of PrPres formation inhibition in ScNB cells. In addition, based on this finding, we proved that this interaction analysis using the SPR method was useful for screening to identify new candidates of anti-prion compounds. Three different *in vitro*

screening assays have been reported recently. One is yeast based,[27] one uses ScN2a cells,[10] and the other is based on fluorescence correlation spectroscopy.[28] These assays are suitable for high-throughput screening of large compound libraries to identify novel lead molecules. The SPR method reported here, which easily assayed interactions between compounds and PrP molecules within less than 3 min per compound, is applicable to high-throughput *in vitro* assay for screening of large compound libraries if more highly performing SPR machines are used. The usefulness of this method in screening for PrP binding ligands is also reported very recently by other researchers.[29]

Two chemicals, ThT and diazepam, showed high binding response but did not inhibit PrPres formation within a non-toxic dose range. Of them, ThT exhibited very low or no affinity with PrP121—231 but the next highest binding response to QC. This suggests that ThT might interact with PrP121—231 non-specifically. For diazepam, similar non-specific interaction with PrP121—231 might be occurred, or the interaction might be specific but unrelated to conversion to PrPres. These inferences, however, remain unsupported by other experimental results obtained here.

On the other hand, such high-affinity compounds as CR and PcTS showed large amounts of binding to PrP121—231. One possible interpretation for this is that the compounds might have two or more binding sites per molecule. In fact, structure–activity relationship analysis for these symmetrical compounds indicates that either half of the molecule has anti-prion activity (Doh-ura K, unpublished data), and their sensorgrams looked very similar to those of anti-PrP antibodies (data not shown). The other is that the compounds might self-assemble to interact with the PrP molecule. It has long been known that CR and many other bis-azo dyes self-assemble in water solutions, and this property is proposed to associate with binding capability.[30]

Instead of the full length of mouse PrP, a carboxy-terminal domain of mouse PrP (PrP121—231) was used in the study because of instability of the full length PrP during the experiment. This carboxy-terminal domain is the only autonomous folding unit of PrP with a defined three-dimensional structure[19,23,24] and contains epitopes recognized by a majority of antibodies bearing anti-prion activity.[31—37] Taken together with our findings suggesting that most of anti-prion compounds might exert their effects by interacting with this domain, targeting the carboxy-terminal domain should not necessarily be either inefficient or inappropriate for looking for new anti-prion compounds.

In conclusion, our study indicated that most anti-prion compounds tested here interacted with and had an affinity for recombinant PrP121—231. The SPR binding response to the PrP121—231 correlated with the anti-prion activity in ScNB cells. These observations will allow further discovery of new classes of anti-prion compounds using the SPR assay.

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