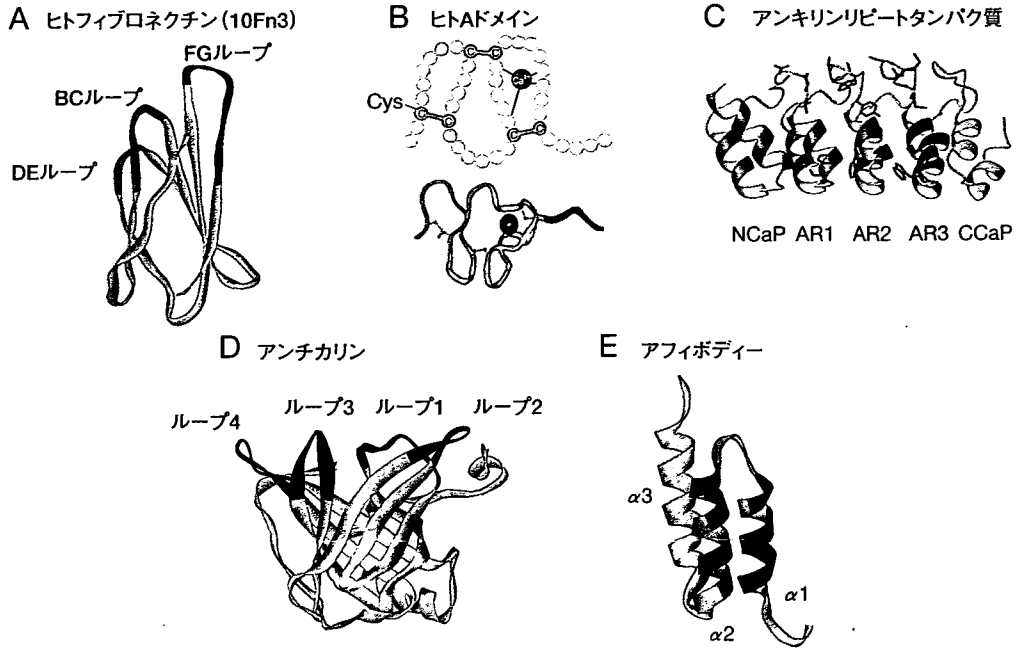

図2. 低分子化抗体の分子骨格

A：モノボディーの分子骨格となるフィブロネクチン10Fn3ドメインの立体構造（PDB ID[注2]；1TTF）．逆平行β構造のサンドイッチ構造と，BC，DE，FGという名称の3本のループ構造が，IgのV領域の構造と類似している．このBC，DE，FGと呼ばれる3本のループのアミノ酸配列をランダマイズすると，その領域が抗体のCDRとして機能する．

B：アビマーの分子骨格となるヒトAドメインタンパク質．6個のシステイン（Cys）残基（橙色）により3個のS-S結合（橙色線）が形成される．分子骨格形成に関与するアミノ酸残基（青色），Ca^{2+}が配位する4アミノ酸残基（黄色）を除く部分をランダマイズした．Silverman J, et al: Nat Biotechnol (2005) 23: 1556-1561より改変．

C：アンキリンリピート（AR）タンパク質．33アミノ酸残基から成るARタンパク質が積み重なった構造を持つ．ARタンパク質のN末端とC末端にはそれぞれNCap（N-terminal capping AR）とCCap（C-terminal capping AR）と呼ばれるARがあり，NCapとCCap間に，デザインされたARタンパク質が組み込まれる．リピート数が2と3のものがライブラリーとして作製されている．Binz HK, et al: Nat Biotechnol (2004) 22: 575-582より改変．

D：アンチカリンの立体構造（PDB ID；1KXO）．アンチカリンの分子骨格となるリポカリンファミリーの分子では，8本の逆平行β鎖が並んだ筒状のタンパク質構造（バレル構造）が高度に保存されている．β鎖の間には4本のループ構造（赤色）が形成され，これらのループ構造がリガンドポケットとして機能する．ライブラリーではこれらのループ構造に変異が導入された．

E：プロテインAのZドメインが分子骨格となるアフィボディーの立体構造（PDB ID；1Q2N）．ヘリックス1（α1）とヘリックス2（α2）の13残基に変異が導入される．

性を示す[13]．さらにヒト血清中には0.1mg/mlという高濃度でFn3ドメインが含まれるという報告もあり，10Fn3を鋳型とするモノボディーのタンパク質医薬としての実用化が期待される．免疫グロブリン（Ig）のV領域の構造に似た10Fn3ドメインを分子骨格として（図2A），Koideらは，10Fn3のBCとFGループの配列をランダマイズしたファージライブラリーを作製，このライブラリーからユビキチンに特異的なモノボディーを単離し，10Fn3ドメインのループ構造にランダムな配列を挿入することで，人工抗体を作製することに成功している[12]．その後Koideらは，10Fn3ドメインの①システイン（Cys）残基を含まない，②金属イオンの結合部位が存在しないという2つの性質を利用して，酵母two-hybridシステムの活性化ドメインに10Fn3ライブラリー，結合ドメインに標的となるエストロゲン受容体α（estrogen receptor α：ER α）のリガンド結合ドメインを導入し，ER αリガンドの存在下，非存在下で選別を行い，細胞内のER αのコンフォメーション変化を識別するプローブを作製し，10Fn3ドメインが細胞内プローブとして有用であることを明らかにした[14]．Xuらも10Fn3ドメインを分子骨格として，mRNAディスプレイ技術を用いたライブラリーを作製し，結合力がKd = 10～200nMのヒトVEGF（vascular endothelial growth factor）受容体2（VEGFR-2）に特異的な10Fn3（AdNectin）を単離，さらにアフィニティーマチュレーションを行い結合力がKd = 0.06～2nMのAdNectinを作製した

注2 256ページ参照．

[13]. VEGFR-2に特異的AdNectinは現在，癌治療薬(AdNexus)として，第Ⅰ相試験が行われている．10Fn3ドメインを用いたモノボディーライブラリーの作製からスクリーニングまでを記載したプロトコールが2007年にKoideらにより報告されている[15].

2. アビマー (Avimer)

アビマーは，P. Stemmerらが報告した新しい抗体様小分子であり，生物界に多数存在する微小タンパク質ドメインのうちAドメイン（～35アミノ酸；4kDa）をもとに作製された(図2B)．Aドメインは様々なヒト細胞表面受容体の細胞外部に存在し，低分子，タンパク質，ウイルスなど100種類以上の異なる標的分子と結合することが報告されている．彼らは，Aドメインのうち分子骨格の形成に関与する2残基と，ジスルフィド(S-S)結合を形成する6つのシステイン残基，およびCa^{2+}が配位する4つのアミノ酸残基はそのままとし，その他の配列をランダマイズしたファージライブラリーを作製した[16]．アビマーは複数の結合ドメインを持つマルチドメインタンパク質であり，選別のたびに新規の結合ドメインを付加することで異なるエピトープを認識するマルチマーを作製できる．Aドメインは最大八量体まで効率良く大腸菌で発現させることができ，高収率（＞1.4g/l）で活性のあるアビマーを得ることができる．また構造的に非常に安定であり，緩衝液中では数週間，血清中では数日間その構造を保持できる．しかし，アビマーはその小ささゆえに生体内で急速にクリアランスされてしまうという問題がある．P. Stemmerらは，この問題を克服するためにIgG結合ドメインをアビマーに結合することにより半減期を90時間まで延長した[16].

人工の分子を臨床応用する際には免疫原性が低くなくてはならない．アビマーはサイズが小さくまた，S-S結合を多く持っているために免疫原性は低くなっている．マウスにアビマーを免疫し，その後血清中の抗アビマー抗体の有無を調べたが検出できなかった[16]．ジスルフィド密度の高い他の分子に関する研究から，このような分子は抗原提示細胞によってプロセシングされにくいので，抗原提示の効率が低くなると考察している．

実際にこれらの手法を用いて，Stemmerらは，cMET〔肝細胞増殖因子（hepatocyte growth factor；HGF）の受容体〕，CD40L (CD40 ligand)，IL-6，CD28，BAFF (B cell-activating factor belonging to the TNF family) に結合するアビマーを単離した[16]．抗IL-6アビマーは，3つのAドメインから成る三量体であり，IL-6に対する結合力がKd＝0.1nMオーダーであった[16]．このアビマーは市販されている抗体と同等，またはそれ以上の細胞増殖阻害活性を有していた．さらに，マウスを用いた実験においてもこのアビマーはIL-6が誘導する血清アミロイドAの産生を阻害した[16]．これらの結果はアビマーを用いた自己免疫疾患や炎症の治療薬としての可能性を示しており，現在クローン病治療薬として第Ⅰ相試験が行われている．

3. アンキリン (Designed Ankyrin Repeat Proteins：DARPins)

アンキリンリピート（Ankyrin repeat；AR）とは生物界に広く見られる分子骨格で，このリピートにより共通の構造的ドメインを形成し，強力なタンパク質間相互作用を媒介する．A. Pluckthumらはこの ARを利用して還元条件下でも酸化条件下でも機能し，細胞質内でも機能する標的化分子の構築を試みた[17]．ヒトARタンパク質は，33アミノ酸残基が作るβターン，2本の逆平行αヘリックス，ループの構造ユニットが積み重なった繰り返し構造を有する（図2C)．前述の10Fn3ドメインと同じくシステイン残基を含まず，バクテリアを用いた大量発現系の構築が容易で，熱力学的に安定な分子である．分子構造は，Igの構造とは異なるが，Binzらは，ARタンパク質のループ構造に抗体のCDRを移植するという発想でランダムな配列を挿入したライブラリーを作製し，マルトース結合タンパク質およびキナーゼをモデル抗原として，結合力がKd＝1nMオーダーの特異的アンキリンを単離し，ARタンパク質のループ構造にランダムな配列を挿入することで，人工抗体を作製した[17]．その後，Zahndらは，ヒトEGFR2 (human epidermal growth factor receptor 2；Her2) を標的として，EGFR1と交差反応しないHer2特異的DARPinのほか，ハーセプチン®(一般名；トラスツズマブ）と同じエピトープのDARPin，結合力がKd＝7.3nMのDARPinを作製したことを報告した[18]．今後の診断，治療への利用が期待される．また，Kaweらは，タバコエッチウイルス（TEV）の繁殖に必須のNIa^{pro}プロテアーゼを標的として，特異的DARPinの選別を行った後，酵母two-hybridシステムを改変した手法を用いて，細胞内でNIa^{pro}プロテアーゼの活性を阻害するDARPinの作製し，DARPinは細胞内で機能できる低分子抗体であることを示した[19].

4. アンチカリン (Anticalin)

リポカリンファミリーの分子（160～180アミノ酸残基）は，主にビタミン，ステロイド，代謝産物のトランスポーターとして機能し，細菌からヒトに至るまで，リガンド輸送から細胞分化まで様々な生物機能のために用いられている分子である．また，バレル構造が高度に保存されている（図2D)．リポカリンファミリーに属する分子を分子骨格に持つ

人工抗体をアンチカリンと呼び，A. Skerraらにより報告されている．彼らはオオモンシロチョウ由来のリポカリンであるビリン結合タンパク質（bilin-binding protein；BBR）を分子骨格として，BBRの4本のループ構造にランダムな配列を挿入したファージライブラリーを作製し，フルオレセイン，ジゴキシゲニンといった小分子に特異的なアンチカリンを単離した[20]．さらに彼らは，ヒト由来のリポカリンであるヒトアポリポタンパク質D（apolipoprotein D；ApoD）を分子骨格としたファージライブラリーを作製し，ヒトヘモグロビンを標的としてスクリーニングを行い，ヘモグロビンに特異的なリポカリンを単離した[21]．すでにCTLA-4（cytotoxic T-lymphocyte-associated protein 4），VEGFに特異的なアンチカリンが確立され前臨床試験が予定されている[22]．

5. アフィボディー（Affibody）

アフィボディーは，ブドウ球菌由来のプロテインA（Staphylococcal protein A；SPA）のIgG結合ドメインの1つであるZドメインを分子骨格として作製した人工抗体である（図2E）．Zドメインは，58アミノ酸残基から成り，3本のヘリックスが束状になった構造で，Nordらは，このZドメインのhelix1とhelix2の溶媒と接触する分子表面の13残基に変異導入したファージディスプレイライブラリーを作製し，Taq DNAポリメラーゼ，ヒトインスリンを標的に，結合力がKd＝1 μMのアフィボディーを単離したことを報告している[23]．またCD28，Her2，アミロイドβペプチドをターゲットに特異的アフィボディーが単離され[24)~26)]，研究用試薬として市販されている．

おわりに

体内に投与しない低分子化抗体の分子群の構築では，まさに遺伝子の切り貼り細工による自由自在なデザインが可能である．しかし体内に投与する医薬品の場合，これらの分子群は“高度の抗原特異性の保持”と“免疫原性をまったく示さないこと”が最も重要でかつ必須な特性であると考えられる．他の特性，例えば①k_a（結合速度定数）/k_d（解離速度定数）の結合力の制御，②pHや熱力学的安定性，プロテアーゼ抵抗性，③高度の溶解性を有し凝集性のないこと，などは検討すべき重要な問題であるが，薬剤学的な他の手法も大きく貢献できる．④抗体遺伝子の発現や組換え体のフォールディング，⑤Fcエンジニアリングによるエフェクター機能のデザインも開拓されるべき問題である．しかし現在開拓されつつある分子群を観ていると，①安全でかつ強力な効果を示すこと，②高度の安定性などの薬理動態プロフィールを有すること，③投与法・服薬の簡便であること，④遺伝子組換え微生物を用いた生産の低コスト化が達成されることなどの課題は，すでに荒唐無稽の話ではなく，克服可能な射程内の問題になっている．抗体エンジニアリングの革命はまだ続いている．この激流に飛び込んだ研究者たちが，“低分子化抗体”のこのようなハードルをどのように越えるのか，また，今後どのように新たなデザインを生み出していくのかを注視したい．

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Immunization with recombinant bovine but not mouse prion protein delays the onset of disease in mice inoculated with a mouse-adapted prion

Daisuke Ishibashi [a], Hitoki Yamanaka [a], Naohiro Yamaguchi [b], Daisuke Yoshikawa [b], Risa Nakamura [b], Nobuhiko Okimura [b], Yoshitaka Yamaguchi [d], Kazuto Shigematsu [c], Shigeru Katamine [b], Suehiro Sakaguchi [a,b,d,*]

[a] *PRESTO Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan*
[b] *Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*
[c] *Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*
[d] *Division of Molecular Cytology, The Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*

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Abstract

Host tolerance to endogenous prion protein (PrP) has hampered the development of prion vaccines as PrP is a major component of prions. Indeed, we show that immunization of mice with mouse recombinant PrP elicited no prophylactic effect against a mouse-adapted prion. However, interestingly, mice immunized with recombinant bovine PrP developed the disease significantly later than non-immunized mice after inoculation of a mouse prion. Sheep recombinant PrP exhibited variable prophylactic effects. Mouse recombinant PrP stimulated only very weak antibody responses. In contrast, bovine recombinant PrP was higher immunogenic and produced variable amounts of anti-mouse PrP autoantibodies. Sheep recombinant PrP was also immunogenic but produced more variable amounts of anti-PrP autoantibodies. These results might open a new way for development of prion vaccines.

Keywords: Prion; Vaccine; Tolerance

1. Introduction

Transmissible spongiform encephalopathies or prion diseases, including Creutzfeldt–Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals, are a group of devastating neurodegenerative disorders transmitted by unconventional infectious agents, the so-called prions [1,2]. Many lines of recent evidence suggest that BSE prions could orally transmit to humans via contaminated food, causing new variant type CJD in young people [3–5]. It was also recently reported that blood transfusion could be a risk factor for prion transmission in humans, causing subsequent CJD in recipients [6,7]. However, no prophylactic measures against the transmission of prions have been developed.

Prions are thought to be mainly composed of the proteinase K (PK)-resistant, amyloidogenic isoform of prion protein, designated PrP^{Sc}, which is generated by conformational conversion of the normal cellular isoform of PrP (PrP^{C}) via unknown post-translational modifications [1,2]. PrP^{C} is

* Corresponding author at: Division of Molecular Cytology, The Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Tel.: +81 88 633 7438; fax: +81 88 633 7440.
E-mail address: sakaguch@ier.tokushima-u.ac.jp (S. Sakaguchi).

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a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein most abundantly expressed in neurons [1,2]. PrP is therefore a plausible target molecule for the development of prophylactic measures against prions. Gabizon et al. previously reported that polyclonal antibodies against PrP could reduce the infectivity of hamster-adapted prions by a factor of 100 [8]. Heppner et al. [9] recently showed that mice transgenically expressing anti-PrP monoclonal antibody, 6H4, were resistant to the disease after intraperitoneal inoculation of mouse-adapted scrapie RML prions. White et al. also demonstrated that two other anti-PrP monoclonal antibodies, ICSM 18 and 35 could prevent prion infection in mice by passive immunization [10]. This successful prevention of prion infection by anti-PrP antibodies indicates that active immunization or vaccination against PrP could be a promising prophylaxis against prion transmission.

In the present study, we immunized BALB/c mice with recombinant mouse, bovine, and sheep PrPs and thereafter intraperitoneally challenged these immunized mice with a mouse-adapted prion. Immunization with mouse recombinant PrP showed no prophylactic effect against the prion infection in mice. Instead, the immunization appeared to exacerbate the infection. In contrast, mice immunized with bovine recombinant PrP exhibited slightly but significantly prolonged incubation times, compared with those of non-immunized mice. The immunizing effects of sheep recombinant PrP on the infection were variable.

2. Materials and methods

2.1. Expression and purification of recombinant PrP immunogens

DNA fragments corresponding to the mouse PrP residues 23–231 (according to GenBank accession no. M13685), the sheep PrP residues 25–234 (GenBank accession no. U67922), and the bovine PrP residues 25–242 (GenBank accession no. AJ298878) were independently amplified by polymerase chain reaction (PCR) using appropriate primer pairs shown in Table 1. Following sequence confirmation of these PCR products, the fragments were digested with *Bam*HI and *Hin*dIII and inserted into a pQE30 vector (QIAGEN, Hilden, Germany). The pQE30 vector was developed to produce the proteins of interest with a N-terminal 6× His tag.

E. coli (M15) cells were freshly transformed by each plasmid, cultured in LB medium containing 1 mM isopropylthio-β-D-galactoside (IPTG), and collected by centrifugation. The collected cells were lysed using CelLytic B bacterial cell lysis/extraction reagent (Sigma–Aldrich Co., St. Louis, USA) in the presence of deoxyribonuclease I and the lysate was centrifuged at 25,000 × *g* for 10 min. The resulting pellet was suspended in Reagent containing 0.2 mg/ml lysozyme and incubated with occasional shaking at room temperature (RT) for 15 min. Volume of the suspension was then increased by addition of 1:10 diluted Reagent and centrifuged at 25,000 × *g* for 10 min. The resulting pellet was washed 3 times with the 1:10 diluted Reagent, suspended in a lysis buffer (8 M Urea, 10 mM Tris–HCl, 100 mM Na_2HPO_4, pH 8.0) and further purified using a Ni-NTA column (QIAGEN) as recommended in the manufacturer's protocol.

2.2. Purification of recombinant mouse PrP minus a 6× His tag

The DNA fragment corresponding to mouse PrP 23–231 was amplified by PCR using an appropriate pair of primers (Table 1). Following sequence confirmation, this fragment was digested with *Nde*I and *Bam*HI and inserted into a pET11a vector (Novagen, Inc., WI, USA). *E. coli* (BL21) cells were transformed by the resulting plasmid and cultured in LB medium containing 1 mM IPTG. The cells were collected by centrifugation and suspended in buffer (50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, pH 8) containing 300 μg/ml lysozyme. After incubation for 20 min at RT, deoxycholic acid was added to the suspension for another 20 min and genomic DNA was digested with deoxyribonuclease I at RT for 30 min. The resulting extract was again centrifuged at 25,000 × *g* for 20 min and the pellet was solubilized in buffer (8 M urea, 50 mM Tris–HCl, 1 mM EDTA, pH 8). This extract was applied to a CM-sepharose column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and recombinant PrP was eluted using a linear NaCl gradient from 0 to 500 mM in the same buffer.

2.3. Immunization

Purified recombinant PrPs with a 6× His tag were dialyzed against PBS and 100 μg of each recombinant protein were intraperitoneally inoculated into a 4 week-old female

Table 1
The DNA sequences of primers used for constructs

Constructs	Forward primers	Reverse primers
6× His-tagged PrPs		
Mouse PrP23–231	gcggatccaaaaagcggccaaagcctggag	ccaagcttctatcagctggatcttctcccgtcgta
Bovine PrP25–242	gcggatccaagaagcgaccaaaacctggag	ccaagcttctatcaacttgccccctcgttggtaata
Sheep PrP25–234	gcggatccaagaagcgaccaaacctggcg	ccaagcttctatcaacttgccccccctttggtaata
Non-tagged PrP		
Mouse PrP23–231	ggatgccatatgaaaaagcggccaaag	gaggatcctattagctggatcttctccc

Underlined sequences indicate appropriate restriction enzyme sites described in Section 2.

BALB/c mouse (SLC Japan, Shizuoka, Japan) at 2-week intervals together with complete Freund's adjuvant (Difco Laboratories, Detroit, MI) for the first immunization and with incomplete Freund's adjuvant (Difco Laboratories) from the second immunization. Antisera were collected 1 week after the final immunization and stored at −20 °C until used. Mice were cared for in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

2.4. Prion inoculation

Brains were removed from the diseased mice infected with the mouse-adapted Fukuoka-1 prion [11] and homogenized to 1% (w/v) in PBS. Aliquots (100 μl) of the homogenate were intraperitoneally inoculated into each mouse 1 week after receiving their fifth immunization with recombinant PrPs.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well immunoplate (Nunc) was coated with 500 ng of purified mouse recombinant PrP without a 6× His tag or other recombinant PrPs with a 6× His-tag by overnight incubation at 4 °C and then blocked with PBS containing 0.05% Tween-20 (T-PBS) and 25% Block Ace (Dainihonseiyaku Co., Tokyo, Japan) at 37 °C for 1 h. To detect specific IgG antibodies, serially 10-fold diluted antiserum was added to the wells for 1 h at 37 °C and unbound antibodies were removed by washing twice with T-PBS. Immune complexes were detected using secondary sheep anti-mouse IgG antibodies conjugated with HRP (Amersham Biosciences), 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and 0.04% H_2O_2. Anti-PrP antibodies titers were determined using colorimetric values expressed at 405 nm.

For ELISA of mouse PrP peptides, moPrP90–109, moPrP131–154, and moPrP219–231, 1 μg of each peptide was coated on a 96-well immunoplate (Nunc) and similarly subjected to the procedures described above except for using 3,3′,5,5′-tetramentylbenzidine (Pierce, Rockford, IL) instead of 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and detecting signals at 450 nm instead of 405 nm. The peptides (>70% purity) were purchased from Sigma–Aldrich Japan K.K. (Hokkaido, Japan).

2.6. Constructions of expression vectors for mouse, sheep, and bovine PrP^C

The DNA fragment encoding full-length mouse PrP^C was amplified by PCR with a sense primer (5′-tcggatcc-agtcatc**atg**gcgaaccttggc-3′; the underlined sequence, a *Bam*HI site; the bold sequence, a start codon) and an antisense primer (5′-cctctagacc**tca**tcccacgatcaggaaga-3′; the underlined sequence, a *Xba*I site; the bold sequence, a stop codon) using a cloned mouse genomic DNA as a template. The DNA fragments for sheep and bovine PrP^C were similarly amplified with a sense primer (5′-tcggatccagtcatc**atg**gtgaaaagccac-3′; the underlined sequence, a *Bam*HI site; the bold sequence, a start codon) and an antisense primer (5′-cctctagacc**tat**cctactatgagaaaaa-3′; the underlined sequence, a *Xba*I site; the bold sequence, a stop codon) using a cloned bovine PrP cDNA and a cloned sheep PrP genomic DNA as a template, respectively. After confirmation of the DNA sequences, each DNA fragment was digested by *Ban*HI and *Xba*I and introduced into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

2.7. Immunoblotting of eukaryotic PrP^C

African green monkey kidney COS-7 cells were transiently transfected by a pcDNA3.1 vector (Invitrogen) inserted with or without the DNA fragment encoding full-length mouse, sheep, and bovine PrP^C using lipofectamin 2000 (Invitrogen) and lysed in lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 300 mM NaCl, 100 mM Tris–HCl, pH 7.5) 3 days after transfection. Proteins were separated by 12% SDS-PAGE and electrically transferred onto an immobilon-P membrane (Millipore, MA, USA). The membrane was incubated with 1:400-diluted antiserum raised against recombinant PrPs in BALB/c mice and secondary sheep anti-mouse IgG antibodies conjugated with HRP (Amersham Biosciences). Immune complexes were visualized using the ECL system (Amersham Biosciences).

2.8. Flow cytometry

Cells were harvested with PBS containing 0.2% EDTA, suspended in BSS buffer (140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1 mM $CaCl_2$ pH 7.0), and incubated with 100-fold diluted antisera for 30 min on ice. The treated cells were then washed three times with BSS buffer, reacted with FITC-conjugated goat anti-mouse IgG (H+L) (Chemicon International, CA, USA), and analyzed by FACScan (Becton Dickinson, NJ, USA).

2.9. Statistical analysis

Logrank test was used for analysis of the incubation times between mice immunized with and without recombinant PrPs. Colorimetric data from ELISA were subjected to one way ANOVA followed by Tukey–Kramer multiple comparison test.

3. Results

3.1. Different effects of immunization with recombinant mouse, sheep, and bovine PrPs on mouse-adapted prion in mice

We intraperitoneally immunized BALB/c mice with purified recombinant mouse, sheep, and bovine PrPs (100 μg/mouse) five times at 2-week intervals and intraperitoneally inoculated a mouse-adapted Fukuoka-1 prion into

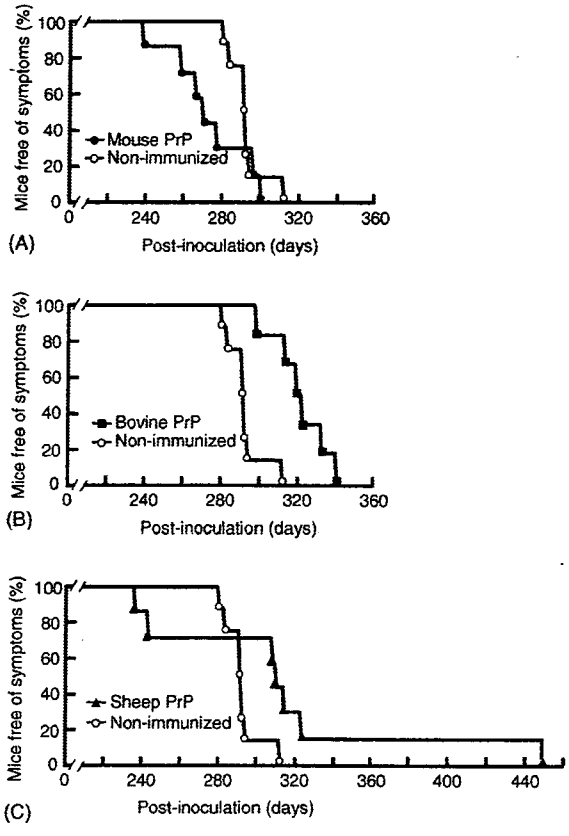

Fig. 1. Incubation times in mice immunized with mouse (A), bovine (B), and sheep (C) recombinant PrPs after intraperitoneal inoculation of a mouse-adapted Fukuoka-1 prion. (A) Incubation times in mice immunized with mouse recombinant PrP ($n=7$) and in non-immunized mice ($n=8$). No prophylactic effect from immunization with mouse recombinant PrP was detected. Instead, incubation times seemed to be shortened, compared with those of non-immunized mice. (B) Incubation times in mice immunized with bovine recombinant PrP ($n=6$) and in non-immunized mice ($n=8$). The immunized mice developed the disease with significantly delayed onset ($p=0.0008$, Logrank test). (C) Incubation times in mice immunized with sheep recombinant PrP ($n=7$) and in non-immunized mice ($n=8$). Except for two of the immunized mice, the other five mice showed extended incubation times compared to non-immunized mice.

the immunized mice 1 week after the final immunization. Non-immunized BALB/c mice developed the disease 291 ± 10 days post-inoculation (p.i.) (Fig. 1). Immunization with mouse recombinant PrP had no prophylactic effect against the disease. The immunized-mice succumbed to the disease at 269 ± 22 days p.i. (Fig. 1A). No significant difference in the incubation times could be detected between the mice immunized with and without mouse recombinant PrP ($p=0.22$, Logrank test), but incubation times of the immunized mice appeared to be shortened compared with those of the non-immunized mice. In contrast, mice immunized with recombinant bovine PrP showed significantly delayed onsets at 322 ± 15 days p.i., compared with non-immunized mice ($p=0.0008$, Logrank test, Fig. 1B). Immunization with recombinant sheep PrP showed variable effects against the prion. Five out of seven immunized mice developed the disease with prolonged onset (Fig. 1C). Two remaining mice became sick at 239 and 246 days p.i., as early as mice immunized with mouse recombinant PrP began to succumb (Fig. 1C). Accumulation of PrP^{Sc} and pathological changes including vacuolation and gliosis were indistinguishable in the brains of terminally diseased mice (data not shown).

3.2. Bovine and sheep but not mouse recombinant PrP stimulates antibody responses against respective immunogens in mice

To assess the immunogenicity of recombinant bovine, sheep, and mouse PrPs in mice, we investigated antibody responses in the immunized mice. Antisera were collected just before prion infection and each serum of the four to five immunized mice of each group was subjected to an ELISA to detect specific IgG antibodies against respective immunizing recombinant PrPs. In the mice immunized with mouse recombinant PrP, only slightly higher antibody binding expressed as optical density values at 405 nm (OD_{405}) were detected, compared with those of non-immunized mice (Fig. 2A). In contrast, much higher OD_{405} values were observed in the mice immunized with recombinant bovine and sheep PrPs (Fig. 2A). We also performed Western blotting of COS-7 cells transiently expressing mouse, sheep, and bovine PrP^C without a 6× His tag using the antisera. No mouse PrP^C could be detected by the anti-mouse recombinant PrP sera on Western blotting (Fig. 2B). In contrast, all of the anti-sheep and -bovine recombinant PrP sera we used for Western blotting substantially detected sheep and bovine PrP^C expressed in COS-7 cells, respectively (Fig. 2B). However, the signals were variable in intensity with each anti-sheep or -bovine recombinant PrP serum. Three out of four anti-bovine PrP sera showed relatively strong signals of bovine PrP^C but the remaining one exhibited faint signals (Fig. 2B). In the case of anti-sheep PrP sera, one antiserum revealed relatively strong signals but the remaining ones exhibited weak signals (Fig. 2B). These results indicate that recombinant bovine and sheep but not mouse PrP were immunogenic but their immunogenicities were variable in BALB/c mice.

We further carried out fluorescence activated cell sorter (FACS) analyses and found that the antisera against bovine and sheep PrPs also contained various amounts of antibodies capable of reacting with respective native PrP^C transiently expressed on COS-7 cells (Fig. 2C).

3.3. Anti-PrP autoantibodies are variably produced in mice immunized with recombinant bovine and sheep PrPs

We investigated whether the antisera against recombinant bovine and sheep PrPs could crossreact with mouse PrP by carrying out ELISA. The immunizing recombinant PrPs contained a 6× His tag. Therefore, to eliminate immunoreactivity

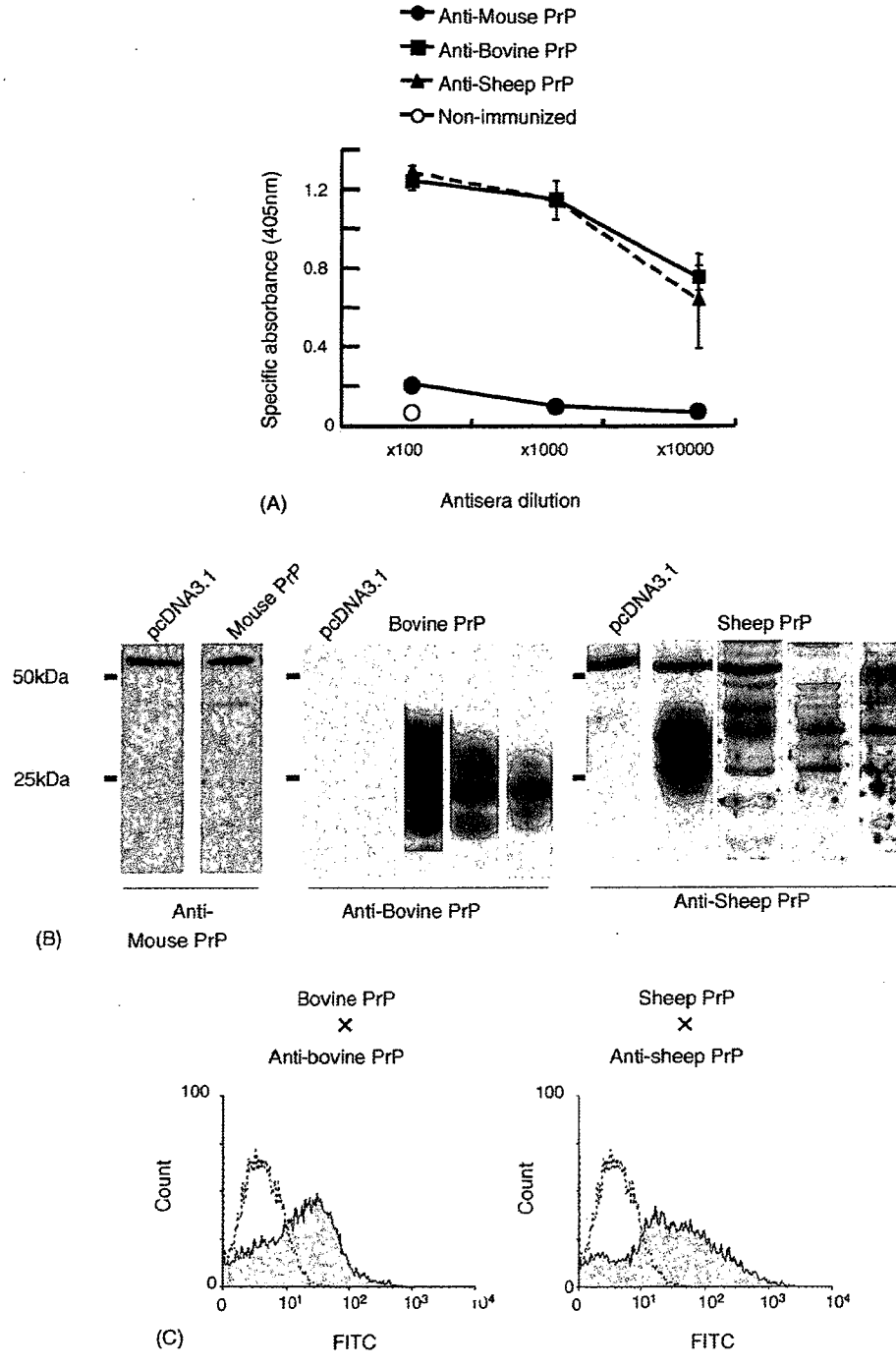

Fig. 2. Antibody responses in mice immunized with mouse, bovine, and sheep recombinant PrPs against the respective immunogens. (A) Each group of at least five mice was intraperitoneally immunized with the purified recombinant PrPs five times at 2-week intervals and anti-PrP IgG antibodies were detected in each serum of the immunized four to five mice of each group by an ELISA against the immunizing antigens. For anti-mouse PrP antibody detection, purified mouse recombinant PrP without a 6× His tag was used instead. (B) Antigenic specificities of each antiserum of the four immunized mice from each bovine and sheep recombinant PrP group were also examined by Western blotting of COS-7 cells transiently transfected with pcDNA3.1 vector alone or with pcDNA3.1 encoding each PrP^C. (C) FACS analysis of COS-7 cells transiently expressing each PrP^C. The cells transfected with the vector alone (unshaded) and the vector encoding bovine or sheep PrP^C (shaded) were probed by each antiserum.

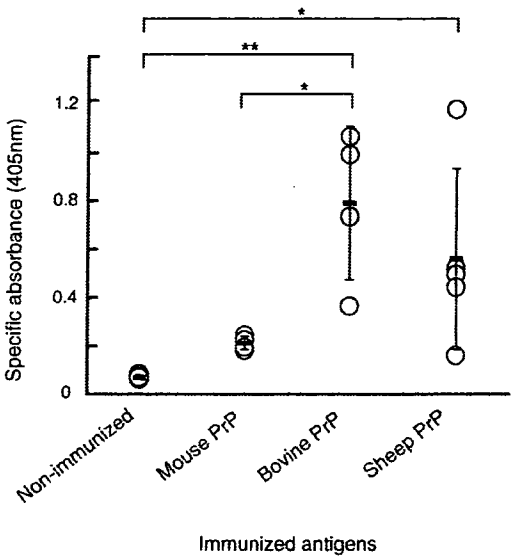

Fig. 3. Anti-PrP autoantibodies in mice immunized with recombinant mouse, bovine, and sheep PrPs. Each antiserum from four to five mice immunized with respective recombinant PrPs was diluted 1:100 and subjected to ELISA against purified mouse recombinant PrP without a 6× His tag. $^{*}p<0.05$; $^{**}p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

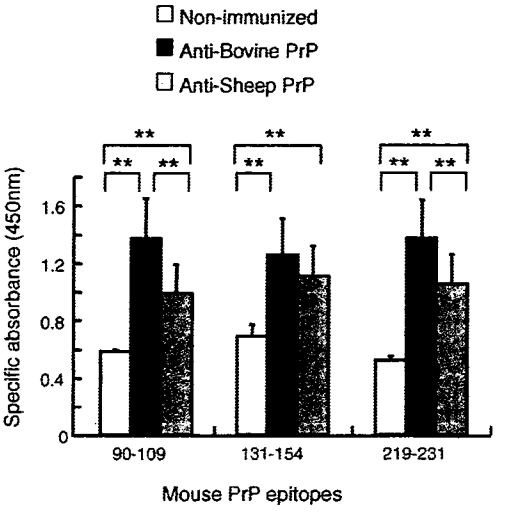

Fig. 4. Recognition of three different mouse PrP epitopes by antisera raised against bovine and sheep recombinant PrPs. The antisera used were collected from four to five mice of each immunization group. Mouse PrP peptides, moPrP90–109, moPrP131–154, and moPrP219–231, positively reacted with antisera 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.

Acknowledgments

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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

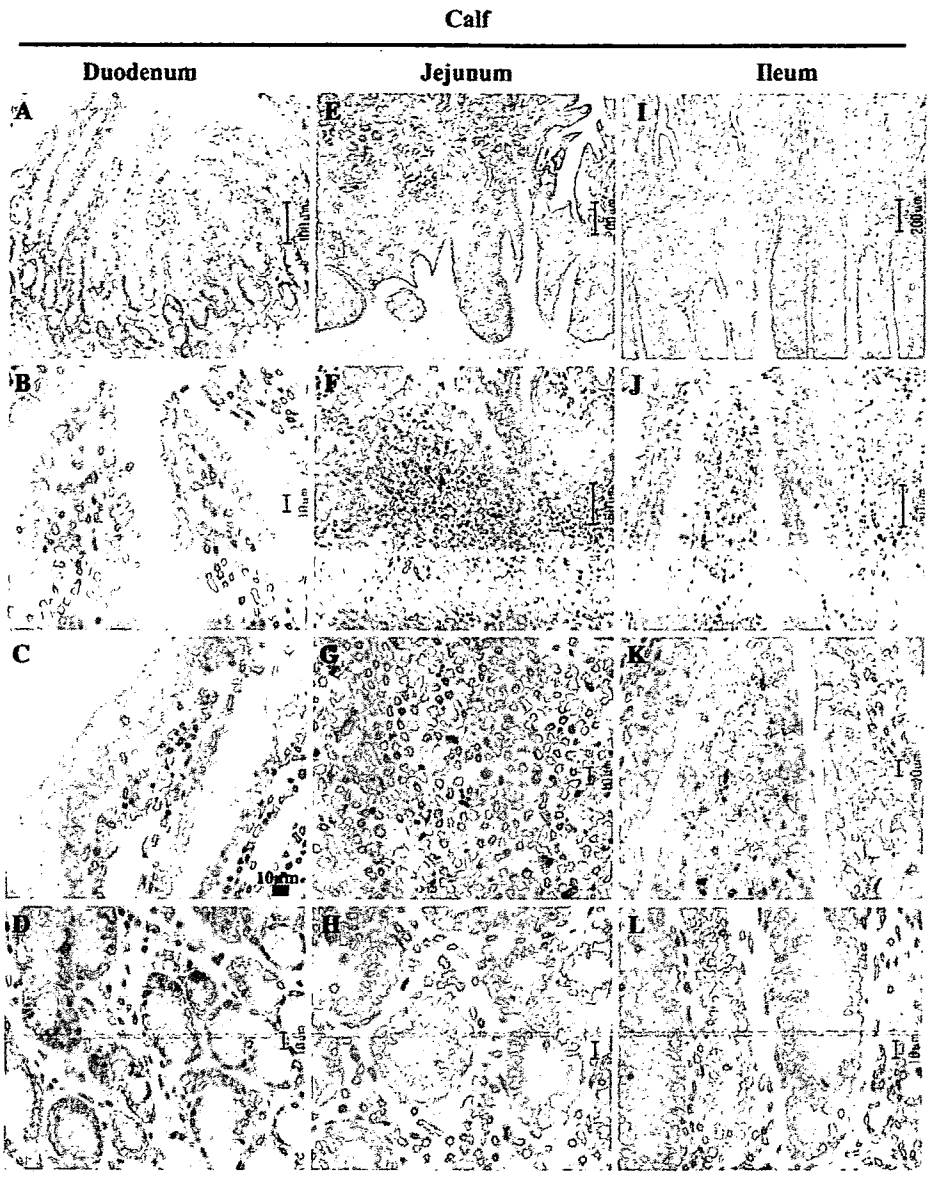

Fig. 1 Localization of PrP^c-positive cells in bovine small intestine. Immunohistochemical micrographs show bovine duodenum (**a–d**), jejunum (**e–h**) and ileum (**i–l**). In the bovine duodenum, PrP^c was clearly observed in some epithelial cells close to the crypt and the lamina propria (**c**, **d**), 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

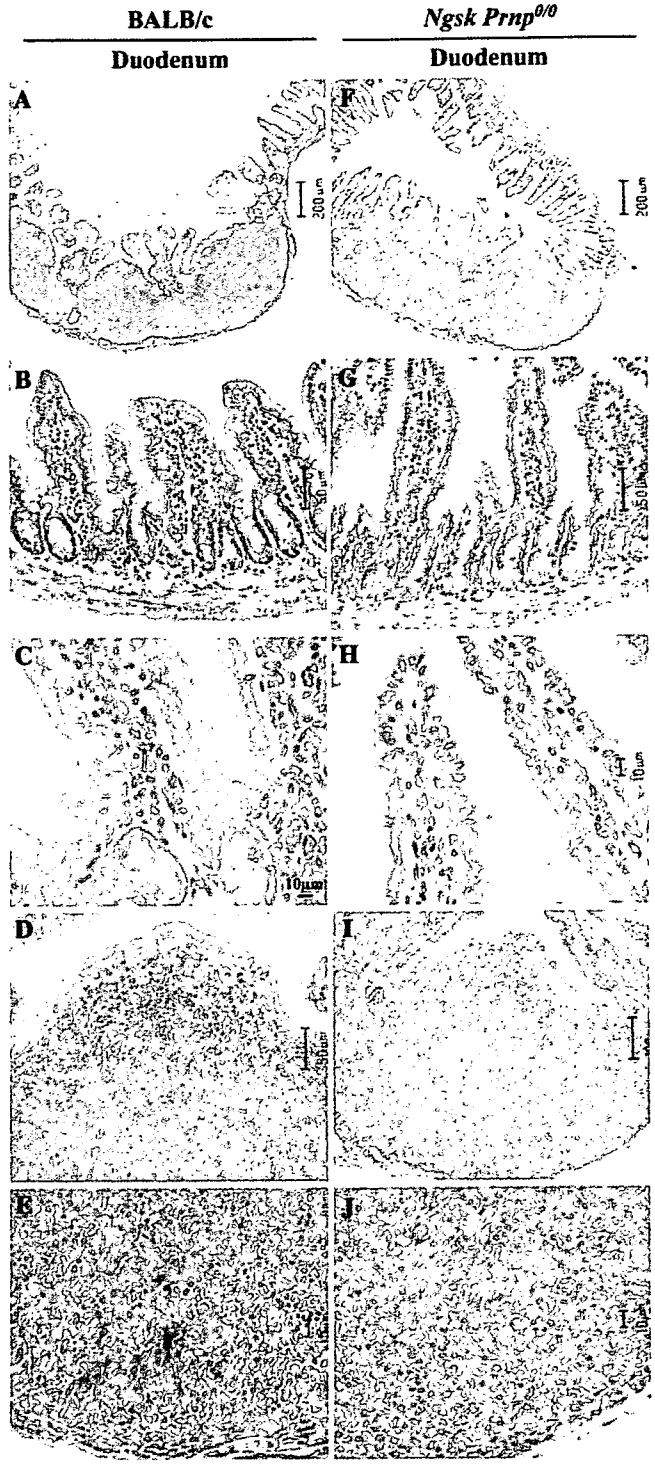

Fig. 2 Localization of PrP^c-positive cells in murine small intestine. Immunohistochemical micrographs show duodenum of BALB/c mice (**a–e**) and prion protein knockout mice (*Ngsk Prnp*$^{0/0}$) (**f–j**). The higher magnification photographs showed that some epithelial cells were clearly stained with 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

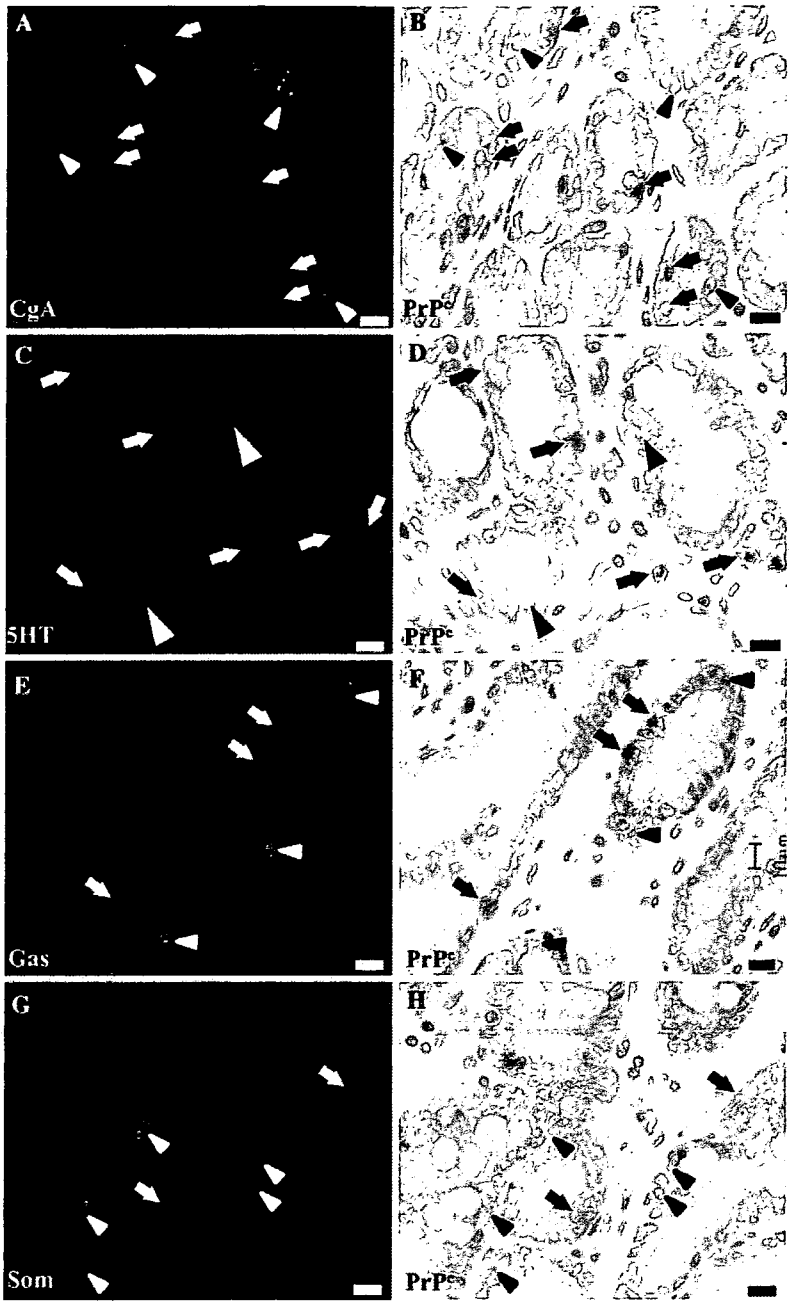

Fig. 3 Dual immunostaining for PrP^c and various neuroendocrine markers in the bovine duodenum. The paraffin sections of the bovine duodenum were performed to the immunohistochemical restaining, using anti-PrP^c antibody and four kinds of antibodies against a neuroendocrine markers. The same section 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

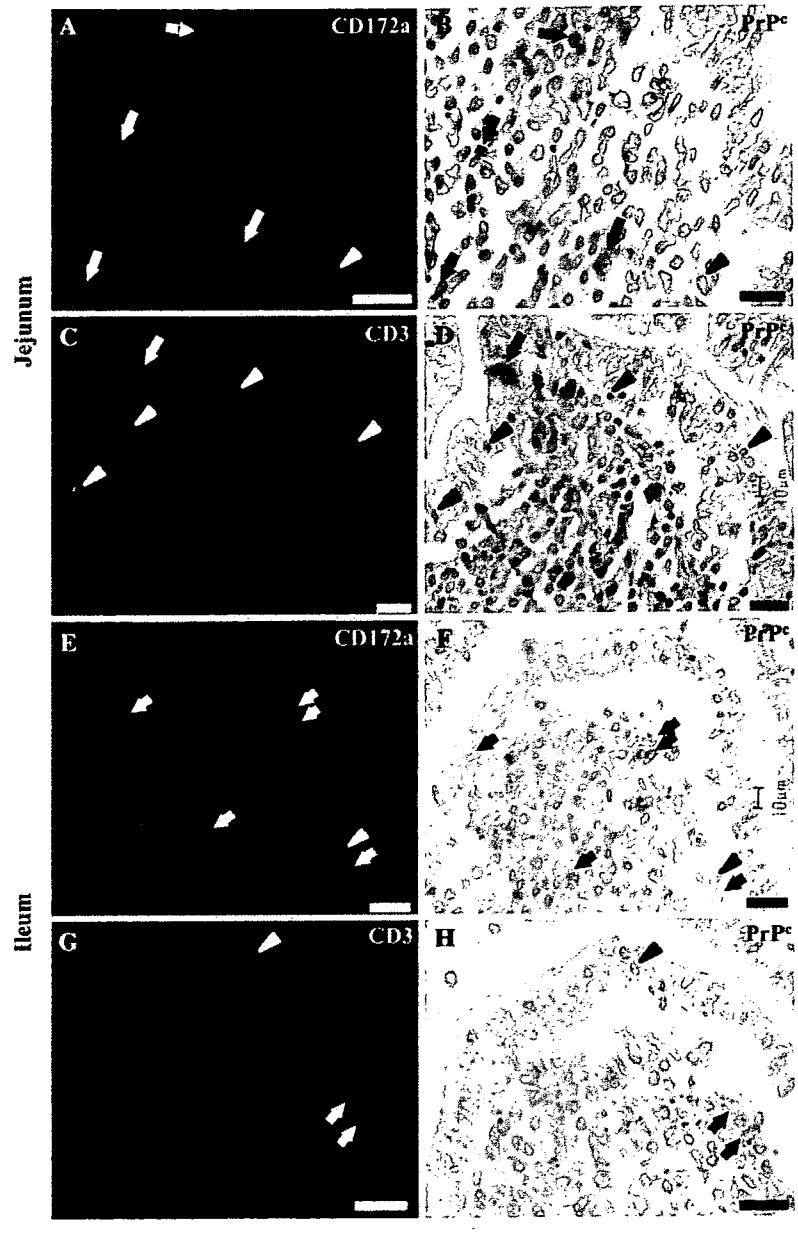

Fig. 4 Identification of PrP^c-positive cells in bovine small intestine. The cryosections of the dome region in bovine jejunal and ileal Peyer's patches were performed to the immunohistochemical restaining, using anti-PrP^c antibody and anti-CD markers. The same section is shown in the *left* and *right* 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 ≈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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