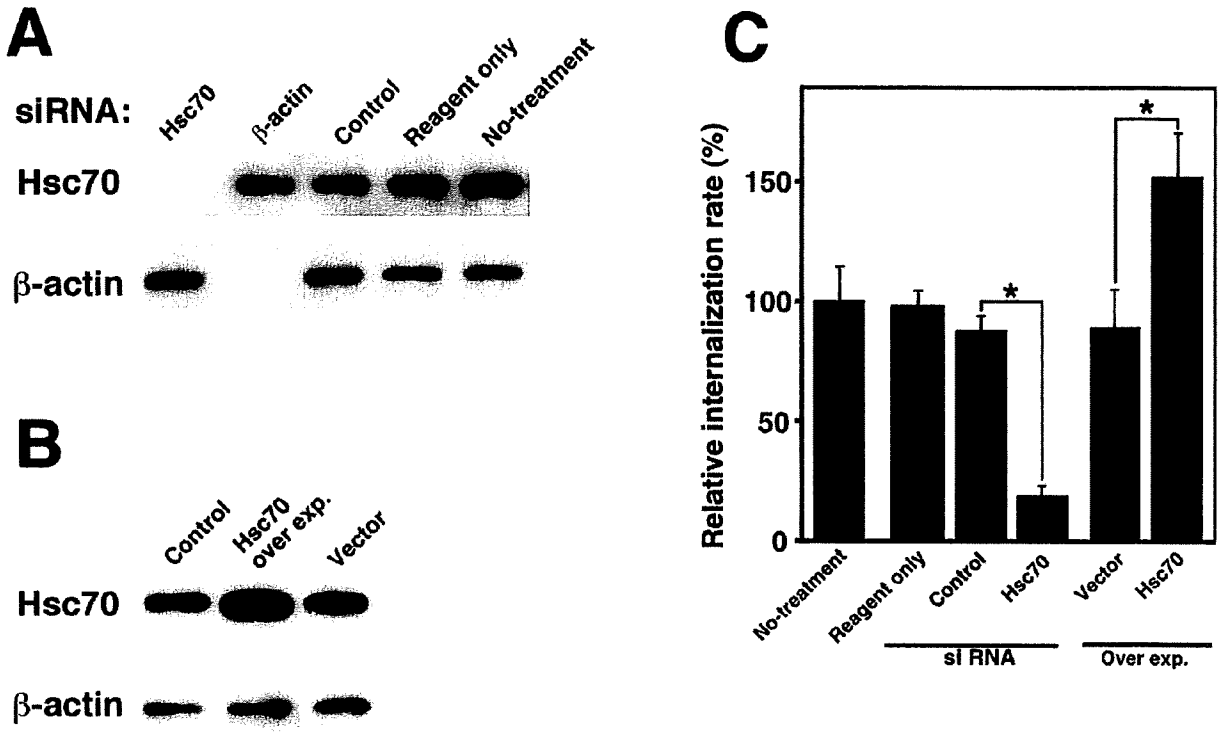

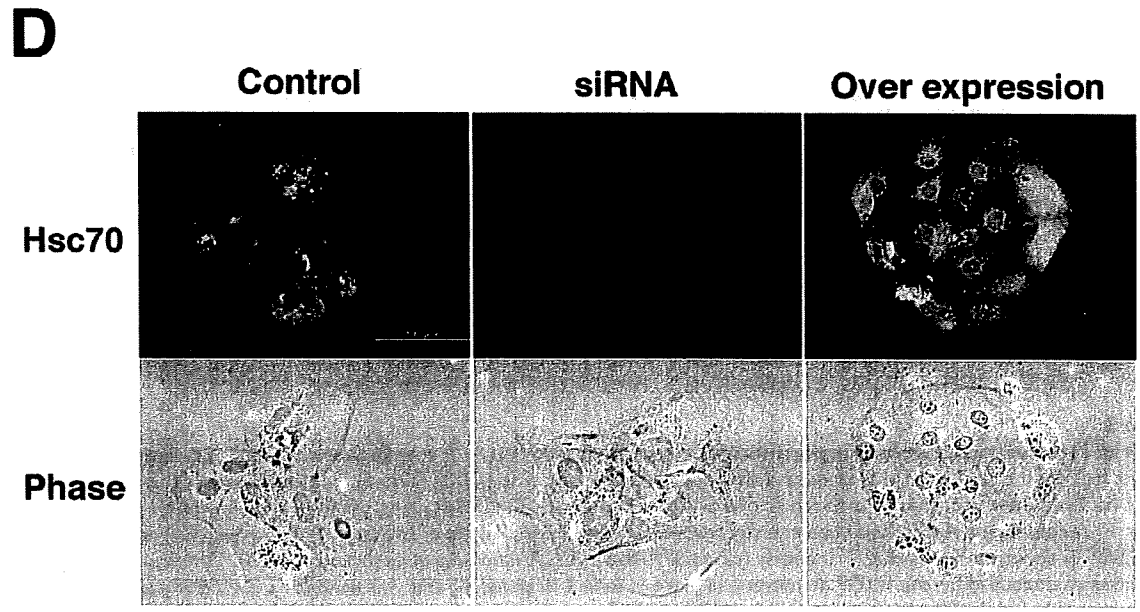

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Effect of depletion and over expression of Hsc70 in TG cells on bacterial internalization. (A) Depletion of Hsc70. TG cells were treated for 48 h with siRNA targeting Hsc70 or without it (reagent only or no treatment), or β-actin or the control (QIAGEN AllStars Negative Control). Expression of the indicated proteins was monitored by immunoblotting. β-actin was used as an internal control. (B) Over expression of Hsc70. TG cells were transfected with or without (control) pcDNA4/TO-Hsc70 or vector only. (C) Bacterial internalization into Hsc70 depleted (siRNA) or over expressed (over exp.) TG cells was studied in a bacterial internalization assay. Lanes correspond to panels A and B. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization into TG cells with (Hsc70) and without siRNA (control), and over expression and the control (vector) are indicated by asterisks (*, $P < 0.01$). (D) Distribution of Hsc70 in non-treated (control), Hsc70 depleted (siRNA), or over expressed (over expression) TG cells. Fluorescence microscopy of stained TG cells with the R2–25 antibody (upper panels) and phase contrast microscopy of the corresponding microscopic fields (lower panels) are shown.

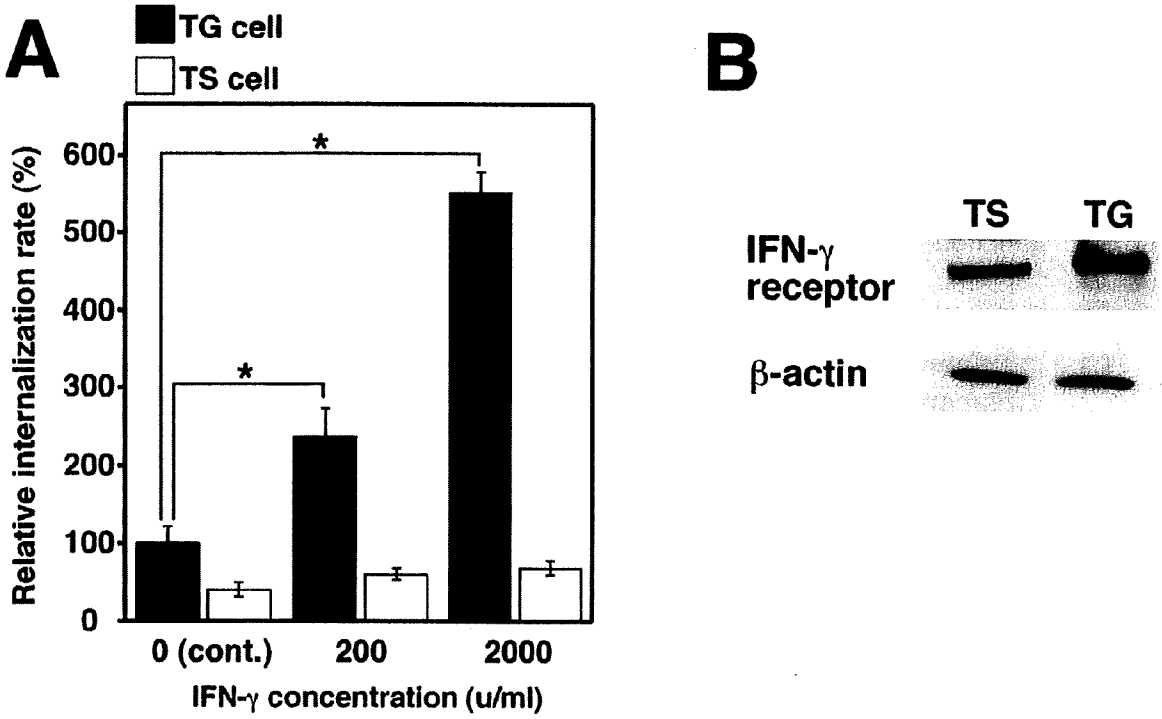

Figure 5
IFN-γ promotes bacterial internalization into TG cells. (A) Bacterial internalization into IFN-γ treated TS or TG cells. *B. abortus* was deposited onto TS and TG cells which were treated with or without (cont.) IFN-γ at the indicated concentrations. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences between bacterial internalization in TG cells with and without IFN-γ treatment are indicated by asterisks (*, $P < 0.01$). (B) Expression of IFN-γ receptor in TS and TG cells. Immunoblot analysis was performed with anti-IFN-γ receptor and anti-β-actin rabbit polyclonal antibody.

by transfecting the Hsc70 expression vector into TG cells. After 48 h, expression levels of Hsc70 were significantly higher than the control levels (Fig. 4B and 4D). The internalization efficiency of *B. abortus* into TG cells in which Hsc70 was over-expressed was significantly higher than the control levels (Fig. 4C).

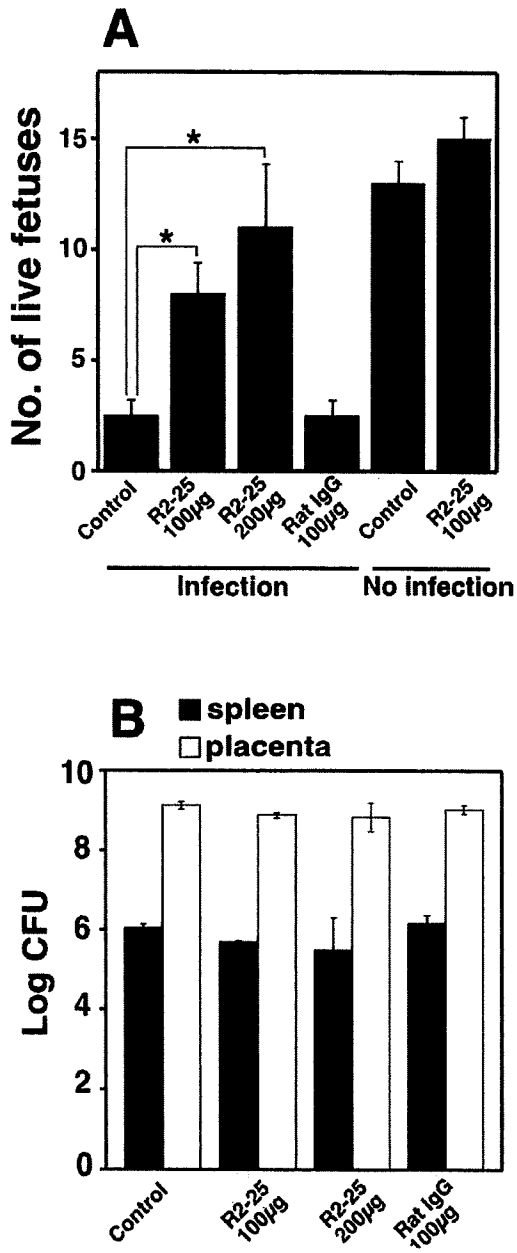

Figure 6

Figure 6
Preventing abortion by inoculating with anti-Hsc70 antibody. (A) Number of live fetuses. Hsc70 was neutralized in the mice by administering with or without (control) an anti-mouse Hsc70 monoclonal antibody (clone R2–25)*in vivo* using 100 or 200 μg of the antibody. The control mice were given 100 μg of normal rat IgG. Statistically significant differences between the untreated control and antibody treated mice are indicated by asterisks (*, $P < 0.01$). (B) Bacterial numbers in spleen and placenta. On day 18.5 of gestation, the placenta and spleen were removed and homogenized in PBS. Tissue homogenates were serially diluted with PBS and plated on Brucella agar in order to count the number of CFU in each organ.

IFN-γ enhances bacterial uptake by TG cells

Since a transient increase in IFN-γ brought about by *Brucella* infection promotes abortion in pregnant mice [10], we investigated the effect of IFN-γ treatment on bacterial internalization and Hsc70 expression in TG cells. IFN-γ treatment significantly increased the internalization efficiency of *B. abortus* into TG cells as their concentration, but had no effect in TS cells (Fig. 5A). To determine whether the enhancement of bacterial internalization by IFN-γ treatment was due to up-regulate Hsc70 expression or not, RNA was isolated from IFN-γ treated TG cells and subjected to RT-PCR. This showed that IFN-γ treatment did not affect Hsc70 expression (data not shown). IFN-γ receptor was expressed in TS and TG cells (Fig. 5B).

Preventing abortion by inoculating pregnant mice with anti-Hsc70 antibody

To determine if abortion is prevented by neutralizing the Hsc70 expressed on TG cells in the mouse placenta, pregnant mice were inoculated with the R2–25 antibody 24 h before infection with *B. abortus*, which was done on day 4.5 of gestation. While there was no change in the number of abortions observed in the non-inoculated mice, there was a significant increase in number of live fetuses in the inoculated mice (Fig. 6A). Inoculation of uninfected pregnant mice with the R2–25 antibody did not affect on pregnancy (Fig. 6A). Upon examining bacterial numbers in the spleen and placenta of infected pregnant mice, it was found that bacterial numbers were similar in both mice inoculated with the R2–25 antibody and those not inoculated with it (Fig. 6B).

Discussion

Previous mouse model studies have shown that *Brucella abortus* specifically replicates in trophoblast giant (TG) cells in the placenta [9,10]. TG cells are polyploid cells that play a crucial role in implantation, in remodeling of the embryonic cavity, and preventing maternal blood

flow to the implantation site [22]. Since *B. abortus* internalizes into TG cells and replicates in them, cell functions are not exhibited completely, which leads to abortion since implantation and placental development are inhibited. Therefore, it is thought that bacterial infection of TG cells is a key event in inducing abortion. To analyze the molecular mechanisms of *B. abortus* infection of TG cells *in vitro*, we used trophoblast stem (TS) cells and TG cells differentiated from TS cells for the infection assay in this study. Although TG cell differentiation is fairly well understood at the morphological and molecular level [23], the role of immune responses in fighting against pathogens of TG cells is poorly understood and in this regard a model of host-pathogen interaction using TG cells would be useful for obtaining new information of the effect of TG cell functions on pregnancy.

Hsc70 has been reported to be present on the surface of several types of cells [24]. In this regard, though Hsc70 congregates on the surface of TG cells, it is present to a much lesser extent on the surface of TS cells (data not shown). This may be a reason that the internalization of *B. abortus* into TG cells was greater than that into TS cells. As Hsc70 and many other factors will be present on TG cells differentiated from TS cells, there is a possibility that other receptors or bacterial uptake-associated molecules may contribute to *B. abortus* infection of TG cells. Little is known about how Hsc70, a protein with no signal sequence for secretion, exits cells by mechanisms other than escape from cells undergoing necrotic lysis. In previous studies, Hsc70 has seen to be released from a late endsomal lysosomal location where it participates in protein degradation [25,26]. Further, the secretion of the Hsp70 family and its association with lipid rafts have also been observed in epithelial cells under normal conditions, and a lipid raft-based mechanism has been suggested for the membrane delivery and release of Hsp70 family [27]. Although receptors for the extracellular Hsp70 family have still not been fully defined, several cell surface receptors have been suggested, such as CD14, CD40, CD91 and scavenger receptor Lox-1 [28-31]. Since it has also been noted that class A scavenger receptor (SR-A) contributes to *B. abortus* infection in macrophages [32], SR-A may be receptors for Hsc70, and the mechanism for *B. abortus* internalization into TG cells may be the same pathway as that for Hsc70 uptake by TG cells. Hsc70 may have a function that is catching antigens and anti-Hsc70 would inhibit binding between Hsc70 and antigens. IFN-γ treatment enhanced bacterial internalization into TG cells and these observations agreed with results obtained in pregnant mice model [10], and thus expression of unidentified receptors against Hsc70 may be upregulated by IFN-γ treatment. IFN-γ should therefore promote internalization of *B. abortus* into TG cells *in vivo* and this would be one of ways in which infectious abortion is induced.

Conclusion

The finding of this study that the anti-Hsc70 antibody prevents abortion caused by *B. abortus* infection is expected to be applied in the development of methods of preventing abortion. Since intracellular bacteria such as *Brucella* replicate in host cells, it is difficult to completely eliminate them from the host through treatment with antibiotics and develop effective vaccines against them. An alternative strategy in treating infection due to *Brucella* would be inhibition of bacterial internalization into TG cells and this could be an effective means of protecting against abortion due to brucellosis. Recently, Carvalho Neta *et al.* reported that *B. abortus* modulates innate immune response by bovine trophoblastic cells [33]. Although the structure of bovine placenta is completely different from mouse placenta, bovine and mouse trophoblastic cells may have similar function in the immune system. However, it is not known whether the mechanism of host-pathogen interaction observed in this study could be used to develop protective methods against other abortion-inducing pathogen infections, and thus further analysis of TG cell function in the immune system will be needed to clarify host defense mechanisms in the placenta and those contributing to the success of pregnancy.

Authors' contributions

MW conceived the study. MW, HS and KW designed the experiments, interpreted the results and worked on the manuscript. KW and MT carried out most of the experimental work. ST, HF and MH participated in cell culture and pathological experiments. HS and MW participated in animal experiments. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Frequencies of *PRNP* Gene Polymorphisms in Vietnamese Dairy Cattle for Potential Association with BSE

Y. Muramatsu[1], Y. Sakemi[1], M. Horiuchi[2], T. Ogawa[3], K. Suzuki[4], M. Kanameda[5], T. T. Hanh[6] and Y. Tamura[1]

[1] Department of Veterinary Public Health, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido, Japan
[2] Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, Japan
[3] Department of Applied Science, School of Veterinary Nursing and Technology, Faculty of Veterinary Science, Nippon Veterinary and Life Science University, Tokyo, Japan
[4] Facultad de Ciencias Veterinarias, Universidad National de La Plata, Provincia de Buenos Aires, Argentina
[5] Japan International Cooperation Agency (JICA), Tokyo, Japan
[6] Department of Veterinary Hygiene, National Institute of Veterinary Research, Hanoi, Vietnam

Impacts

- We have investigated the frequencies of polymorphisms in the prion protein gene (*PRNP*) associated with bovine spongiform encephalopathy (BSE) susceptibility in Vietnamese dairy cattle for the first time.
- We have provided evidence that Vietnamese dairy cattle have a unique genetic background in the *PRNP*.
- We have provided the information on the genetic background in the *PRNP* of dairy cattle in Vietnam, where dairy farming is an emerging industry.

Keywords:
PRNP gene; BSE; cattle; Vietnam; indel polymorphisms

Correspondence:
Yasukazu Muramatsu. Department of Veterinary Public Health, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan. Tel.: +81 11 388 4800; Fax: +81 11 387 5890;
E-mail: y-mrmt@rakuno.ac.jp

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Summary

Since 2004, significant associations between bovine spongiform encephalopathy (BSE) susceptibility in cattle and frequencies of insertion/deletion (ins/del; indel) polymorphisms within the bovine prion protein gene (*PRNP*) have been reported. In this study, we investigated the frequencies of indel polymorphisms within two variable sites, a 23-bp indel polymorphism in the promoter region (23indel) and a 12-bp indel polymorphism in intron 1 region (12indel), in the *PRNP* in 206 Vietnamese dairy cattle and seven Japanese BSE-affected cattle. In Vietnamese dairy cattle, the frequency distributions of del allele and del/del genotypic polymorphisms in the 23indel site, which are thought to be associated with BSE susceptibility, were significantly higher, whereas the frequencies of del allelic and del/del genotypic polymorphisms in the 12indel site, which have been reported to confer BSE susceptibility, were significantly lower. We have provided evidence that Vietnamese dairy cattle have a unique genetic background in the *PRNP* gene in comparison with cattle or sires previously reported in other countries.

Introduction

In Vietnam, dairy farming is an emerging industry. For centuries, cattle have been reared for draught power, making manure and meat production. In the 1920s, dairy cattle breeding was first introduced to Vietnam. However, in the colonial period, dairy production was carried out by French residents. Nationwide expansion of dairy farming occurred in the 1970s. However, the overall production and consumption of dairy products remained very low because of war and the general poor socio-economic situation in the country (Luthi et al., 2006). Thus, until the government economic decentralization policy called 'Doi Moi' was initiated in 1986, there was almost no dairy industry in Vietnam. Since the 1980s, the dairy cow population has been increasing rapidly in both state-

owned and privately owned farming sectors, particularly in the latter sector. The establishment of the Vietnam Dairy Products Joint Stock Company (VINAMILK) in 1976 was followed by the emergence of foreign dairy companies (Dutch Lady Vietnam and Nestlé Vietnam) as well as many smaller domestic or joint venture companies in the 1980s and 1990s. The current development of the dairy industry in Vietnam is rooted in the National Dairy Development Plan for 2002–2010 (NDDP) issued in 2001 (Luthi et al., 2006). Cattle breeding and rearing are now regarded as the most important issues of the NDDP in Vietnam.

Bovine spongiform encephalopathy (BSE) is categorized as one of the transmissible spongiform encephalopathies (TSE), including as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome and fatal familiar insomnia (FFI) in humans and scrapie in sheep and goats. These diseases are also called prion diseases on the basis of the widely accepted hypothesis that the causative agents of TSE are abnormally folded, infectious prion proteins. Bovine spongiform encephalopathy is generally thought to be caused by ingestion of scrapie-infected or BSE-infected meat and bone meal (Wilesmith et al., 1991). Bovine spongiform encephalopathy has also been implicated in the development of variant CJD via consumption of beef from BSE-affected cattle (Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999; Asante et al., 2002; Smith, 2003). To date, several mutations and polymorphisms in the *PRNP* gene of humans and sheep have been reported to be associated with some prion diseases. In humans, a polymorphism, methionine (Met) or valine (Val), at codon 129 of the human *PRNP* coding sequence is known to be a determinant of the disease phenotype in familial CJD and FFI patients having a mutation at codon 178 of the *PRNP*. Moreover, this polymorphism at codon 129 is strongly correlated with susceptibility to variant CJD, as it is known that all variant CJD patients have the homozygous Met/Met genotype, whereas individuals having Val/Val and heterozygous genotypes at codon 129 have not yet been diagnosed as having variant CJD. The association of polymorphisms within the *PRNP* coding sequence and resistance/susceptibility to scrapie has been well documented for sheep (Belt et al., 1995). Of five genetic variants having mutations at codon 136, 154 and 171 in the sheep *PRNP* coding sequence, one variant was present at a high frequency in scrapie-affected sheep, whereas another variant was almost exclusively present in healthy sheep. In contrast, no associations had been reported between polymorphisms in the bovine *PRNP* coding sequence and susceptibility to BSE before 2004.

Recently, novel associations have been shown between susceptibility to classical BSE and specific indel polymorphic sites in two regulatory regions upstream of the coding region of the bovine *PRNP* (Sander et al., 2004; Juling et al., 2006). Sander et al. (2004) showed associations of both allelic and genotypic del polymorphisms in the 23indel site and allelic del polymorphism in the 12indel site with susceptibility to BSE in a study using 48 healthy and 43 BSE-affected cattle. A significant overrepresentation of del allelic polymorphisms at both indel sites was indicated in large number of cattle in a study by Juling et al. (2006). A 23indel site contains a transcription factor RP58-binding site, and a 12indel site contains a transcription factor SP1-binding site. Reporter gene assays have demonstrated an interaction between the two transcription factors and lower *PRNP* expression levels of the allele harbouring ins polymorphisms in both indel sites in comparison with the other allele harbouring del polymorphisms in both indel sites (Sander et al., 2005). Moreover, a haplotype consisting of 12indel polymorphisms and flanking single nucleotide polymorphisms (SNPs) has been reported to correlate with reduced *PRNP* expression and increased resistance to BSE (Kashkevich et al., 2007). In this study, we investigated the frequencies of indel polymorphisms in the *PRNP* in Vietnamese dairy cattle and Japanese BSE-affected cattle. To clarify the genetic background of the *PRNP* in Vietnamese dairy cattle, we compared the frequencies of indel polymorphisms in Vietnamese dairy cattle with those in cattle in other countries.

Materials and Methods

A total of 278 serum samples were collected from dairy cattle in Bavi Village, Hatai Province, Vietnam. All of the bovine serum samples were subjected to extraction of genomic DNA using a commercial kit in accordance with the manufacturer's instructions (QIAamp DNA Mini Kit; Qiagen, Tokyo, Japan). The extracted DNA solution was stored at −20°C until use. Each of the DNA solutions was subjected to PCR for detection of 23indel and 12indel polymorphisms in the bovine *PRNP* (23indel PCR and 12indel PCR respectively). Also, genomic DNA samples obtained from tissues of seven BSE-affected cattle in Japan were subjected to PCR for the same purpose.

The 23indel PCR and 12indel PCR were performed as previously described with slight modification (Sander et al., 2004). Briefly, a 50-μl volume of reaction mixture containing 10–20 μl of each extracted DNA solution was used for the first stages of both PCRs. If an amplified DNA fragment was not detected, 10 μl of the first PCR product was used as a template of the second PCR. The conditions of the reaction mixture and thermal cycling for the second PCR were same as those for the first PCR.

Statistical analyses were performed for data from eight groups of cattle: the Vietnamese dairy cattle we studied,

Japanese healthy Holstein cattle (Nakamitsu et al., 2006), the Japanese BSE-affected Holstein cattle we studied, German healthy cattle of seven breeds and BSE-affected cattle of various breeds (Sander et al., 2004), US sires of 39 breeds (Seabury et al., 2004), and UK healthy and BSE-affected Holstein cattle (Juling et al., 2006). At first, the chi-squared test was performed for the following four categories of data: the frequency distributions of allelic polymorphisms (ins and del) in the 23indel site and in the 12indel site and the frequency distributions of genotypes (ins/ins and del/del) in the 23indel site and in the 12indel site among the eight groups of cattle (each degree of freedom = 7). Then the chi-squared test, the chi-squared test with Yates' correction, or Fisher's exact probability test was used to estimate the associations in bivariate analysis of the categorical data between two groups of cattle: Vietnamese dairy cattle and Japanese BSE-affected cattle, Vietnamese dairy cattle and Japanese healthy cattle, Vietnamese dairy cattle and German healthy cattle, Vietnamese dairy cattle and German BSE-affected cattle, Vietnamese dairy cattle and US sires, Vietnamese dairy cattle and UK healthy Holstein, and Vietnamese dairy cattle and UK BSE-affected Holstein (each degree of freedom = 1).

Results

We detected polymorphic DNA fragments in the 23indel site and in the 12indel site of 206 Vietnamese cattle and seven Japanese BSE-affected cattle. Information on these 213 cattle is summarized in Table 1. The ages of the 206 Vietnamese cattle ranged from 4 to 123 months, and those of the seven Japanese Holstein cattle with BSE detected in four prefectures ranged from 21 to 108 months. Of the 206 Vietnamese cattle studied, 205 cattle were Holstein Friesian (HF) crossbred with Vietnamese indigenous cattle, commonly called Yellow cattle. The remaining one was crossbred with Red Sindhi and Yellow cattle.

The frequency distributions of ins and del alleles in the 23indel site were 15.3% and 84.7%, respectively, for 206 Vietnamese dairy cattle and 7.1% and 92.9%, respectively, for seven Japanese BSE-affected cattle. The frequency distributions each of genotype (ins/ins, ins/del and del/del) in the 23indel site were 1.5%, 27.8% and 70.7% in the Vietnamese cattle, and 0%, 14.3% and 85.7% in the Japanese BSE-affected cattle. On the other hand, the frequency distributions of ins and del alleles in the 12indel site were 52.3% and 47.7% in the Vietnamese dairy cattle, and 7.1% and 92.9% in the Japanese BSE-affected cattle. The frequency distributions of each genotype (ins/ins, ins/del and del/del) in the 12indel site of these cattle were 19.5%, 65.6% and 14.9% in Vietnamese dairy cattle, and 0%, 14.3% and 85.7% in Japanese BSE-affected cattle (Table 2).

Table 1. Vietnamese dairy cattle and Japanese BSE-affected cattle in which results for 23indel- and 12indel-PCRs were obtained in this study

Origin*	Breed	Number	Age (months)
Vietnam/Hatai province	Hol × Yellow (F2 or higher)	126	4–117
	Hol × Yellow (F1)	23	6–123
	Red Sindhi × Yellow	1	60
	UN	56	UN
Japan/Hyogo prefecture	Hol	1	21
Japan/Gunma prefecture	Hol	1	68
Japan/Kanagawa prefecture	Hol	1	95
Japan/Hokkaido prefecture	Hol	4	73, 81, 83, 108

*Origin for Vietnamese dairy cattle means the site at which blood was sampled, and origin for Japanese BSE-affected means the location where BSE was detected.
Hol, Holstein; Yellow, Vietnamese Yellow local cattle.

Statistical analyses of which degrees of freedom were 7 showed significant differences in all of the categorized data (chi-squared values: 52.694 and 53.354 in 23indel site and 122.949 and 118.164 in 12indel site; Table 2). The frequency distributions of the del allele and del/del genotype in the 23indel site observed for Vietnamese dairy cattle closely resembled those observed for Japanese BSE-affected cattle (Table 2). The frequency distribution of the del allele in the 23indel site of Vietnamese dairy cattle was significantly different from the frequency distributions in all other cattle compared in this study except for the Japanese BSE-affected cattle. There was no difference in the frequency distributions of the del/del genotype in the 23indel sites among Vietnamese dairy cattle, Japanese BSE-affected cattle, German BSE-affected cattle and UK BSE-affected cattle (Table 2). The frequencies in 23indel deletion polymorphisms observed for Vietnamese dairy cattle were extremely high in comparison with those observed for all other cattle except the Japanese BSE-affected cattle. The frequency distribution of 23indel polymorphisms in Vietnamese dairy cattle most closely resembled that in Japanese BSE-affected cattle. In contrast, the frequency distributions of the del allele and del/del genotype in the 12indel site observed for Vietnamese dairy cattle were significantly lower than those observed for Japanese healthy Holstein and BSE-affected Holstein, UK healthy Holstein and BSE-affected Holstein and German BSE-affected cattle (Table 2). There was no

Table 2. Comparison of the frequency distributions of 23indel polymorphisms in the promoter region and 12indel polymorphisms in the intron 1 region of the bovine *PRNP* gene among Vietnamese dairy cattle, Japanese cattle, German cattle, US sires and UK cattle

	23indel polymorphisms in promoter region							12indel polymorphisms in intron 1 region							
	Allele			Genotype				Allele			Genotype				
	Frequency %		χ^2-value (degree of freedom)	Frequency %			χ^2-value (degree of freedom)	Frequency %		χ^2-value (degree of freedom)	Frequency %			χ^2-value (degree of freedom)	
Cattle (*n*)	ins	del		ins/ins	ins/del	del/del		ins	del		ins/ins	ins/del	del/del		Reference
All groups (1540; 23indel, 1533; 12indel)			**52.694 (7)**				**53.354 (7)**			**122.949 (7)**				**118.164 (7)**	This study
VN dairy (206)	15.3	84.7	–	1.5	27.8	70.7	–	52.3	47.7	–	19.5	65.6	14.9	–	This study
JPN BSE HF (7)	7.1	92.9	0.211 (1)	0	14.3	85.7	0.123 (1)	7.1	92.9	**9.262 (1)**	0	14.3	85.7	**4.959 (1)**	This study
JPN healthy HF (278; 23indel, 290; 12indel)	21	79	**5.477 (1)**	5	32	63	**4.051 (1)**	26	74	**69.741 (1)**	7	38	55	**55.231 (1)**	Nakamitsu et al. (2006)
GER healthy (48)	43	57	**35.947 (1)**	21	44	35	**36.028 (1)**	49	51	0.155 (1)	21	56	23	0.497 (1)	Sander et al. (2004)
GER BSE (43)	27	73	**6.532 (1)**	5	44	51	1.121 (1)	33	67	**10.969 (1)**	9	47	44	**9.077 (1)**	Sander et al. (2004)
US sires (132)	30	70	**20.763 (1)**	14	32	54	**20.762 (1)**	49	51	0.710 (1)	32	35	33	0.878 (1)	Seabury et al. (2004)
UK healthy HF (276; 23indel, 270; 12indel)	29.2	70.8	**25.464 (1)**	4.7	48.9	46.4	**5.901 (1)**	37.0	63.0	**21.806 (1)**	11.1	51.9	37.0	**22.382 (1)**	Juling et al. (2006)
UK BSE HF (363; 23indel, 350; 12indel)	24.1	75.9	**12.344 (1)**	3.6	41.0	55.4	2.542 (1)	27.9	72.1	**65.943 (1)**	5.1	45.4	49.4	**66.090 (1)**	Juling et al. (2006)

VN, Vietnamese; JPN, Japanese; GER, German; US, American; UK, British; HF, Holstein breed.

Vietnamese daily cattle group consisted of Yellow cattle × Holstein, Yellow cattle × Red Sindhi and unknown breeds. German healthy cattle group consisted of seven cattle breeds, and German BSE-affected cattle group consisted of various breeds (Sander et al., 2004), US sires group consisted of 39 cattle breeds (Seabury et al., 2004). In cases in which a significant difference was detected by statistical analysis, each χ^2-value is shown in bold font.

difference in the frequency distributions of the 12indel deletion polymorphisms among Vietnamese dairy cattle, German healthy cattle and US sires (Table 2). Moreover, there was no cattle group having same combination pattern of frequency distributions of polymorphisms in the 23indel and in the 12indel sites as that shown in the group of Vietnamese dairy cattle (Table 2).

Discussion

Our results suggest that Vietnamese dairy cattle have a unique genetic background in the *PRNP* in comparison with cattle in other countries. Significantly higher frequencies of the 23-bp deletion polymorphism in the promoter region and significantly lower frequencies of the del polymorphism in the 12indel site, which have been shown to be associated with BSE susceptibility, were found in Vietnamese dairy cattle (Sander et al., 2004; Juling et al., 2006). As shown in Table 1, all but one Vietnamese dairy cattle are crossbred between Holstein and Yellow cattle. The remaining cow is crossbred between Yellow cattle and Red Sindhi, an Indian dairy zebu breed introduced to Vietnam by Indian and Pakistani merchants at the beginning of the 20th Century. Between 1995 and 1998, to improve the poor dairy and beef capacity of the indigenous Yellow cattle, crossbreeding between Yellow cattle and Red Sindhi had been facilitated by the government.

In addition to the climate conditions (mostly hot and humid), poor knowledge of dairy cow raising, poor veterinary services and shortage of feed production are main problems for dairy farming in Vietnam. Grass cultivation for livestock purposes is rare in Vietnam (Schmick and Huong, 2003). The indigenous Yellow cattle breed is known to be very well adapted to the harsh environment of Vietnam and to thrive well under poor feeding and management conditions. For example, mortality rate of the calves and cows of the cattle breed are low, even under poor management conditions, and resistance against diseases, particular tick borne diseases and liver fluke, is high. However, the milk productivity of Yellow cattle has been estimated to be very poor. One of the objectives of the NDDP is to produce a new dairy cattle breed for Vietnam that has high milk productivity and is biologically suited to the environmental conditions of the country. To achieve a faster pace of development of dairy farming, approximately 10 000 heads of purebred HF cows were imported between December 2001 and May 2004. On the basis of the NDDP, dairy cattle breeding has carried out to improve the existing stock of dairy commercial farms and to increase the number of commercial farms by importing live cattle and by the use of artificial insemination (AI). Crossbred dairy cattle represent the vast majority of dairy cows. Popular breeds for crossing are HF, Red Sindhi and Yellow cattle (Garcia et al., 2006). 'Holsteinization' of the indigenous cattle stock has been accelerated by using AI (Luthi et al., 2006). In the present study, more than 60% of the 206 Vietnamese dairy cattle were crossbred cattle having at least 75% of Holstein blood. Consequently, the genetic properties of many of the Vietnamese dairy cattle would apply to those of Holstein cattle. Nevertheless, the frequency distribution pattern found in Vietnamese dairy cattle – high frequencies of del polymorphism in the 23indel site and ins polymorphism in the 12indel site – was not found in any other groups of Holstein breed cattle: Japanese healthy and BSE-affected Holstein cattle, and UK healthy and BSE-affected Holstein cattle (Table 2). Although we have no genetic information on the purebred of the Vietnamese indigenous cattle, the frequency distribution pattern of the Vietnamese dairy cattle is probably attributed to those of the indigenous cattle, especially Yellow cattle. Also, it is possible that the frequency distribution pattern of the Vietnamese dairy cattle reflect the genotypes in both indel sites of Holstein cattle used for crossbreeding. To produce dairy cattle with reduced susceptibility to BSE, identification of the genotypes in the indel sites of the cow, bull, and bull semen must precede the crossbreeding.

Reporter gene assays have demonstrated that indel polymorphisms at these sites concern with the expression levels of the bovine *PRNP* gene (Sander et al., 2005; Kashkevich et al., 2007). A functional analysis of the two indel polymorphic sites in the *PRNP* promoter that contain binding sites for RP58 and SP1 transcription factors revealed an interaction between the two transcription factors and lower *PRNP* expression levels of the allele harbouring ins in the 23indel site and ins in the 12indel site compared with the other allele harbouring del in the 23indel site and del in the 12indel site (Sander et al., 2005). Also, it has been reported that 12indel polymorphisms and flanking SNPs are important for *PRNP* expression. A haplotype consisting of 12indel polymorphisms and SNPs associated with the lowest expression level has been shown to correlate with reduced *PRNP* expression and increased resistance to BSE (Kashkevich et al., 2007). These findings support the hypothesis that mutations potentially influencing the level of bovine prion protein expression might also influence incubation time and susceptibility to BSE.

The objectives of the NDDP are as follows: to reach a total dairy cattle population of 100 000 heads by 2005 and 200 000 heads in 2010; to achieve a total milk production of 165 000 metric tons (Mt) in 2005 and 350 000 Mt in 2010, meeting 20% and 40%, respectively,

of the local demand for dairy products; to produce a new dairy cattle breed for the country that has high milk productivity and is biologically suited to the environmental conditions of the country; and to achieve a change in the breed structure of the livestock, and to improve the income and living conditions for household farmers in rural areas (Luthi et al., 2006). Thus, cattle breeding and rearing are important as a main part of the NDDP. A breeding strategy and selection by using dairy cattle with reduced susceptibility to BSE are needed for the dairy industry in Vietnam. The Vietnamese animal feed production sector is now relying heavily on imported feed ingredients. Vietnam imports 60% of materials such as maize, soybean meal, fish meal, meat and bone meal, rice bran and vitamins needed to produce animal feed locally (Wade and Huong, 2005). Attention must be given to the supply of feed in the rearing period so as to prevent cattle from ingesting scrapie- or BSE-contaminated offal. An outbreak of BSE would have a significant impact on the Vietnamese dairy industry, which is still in its infancy. Preparations for an outbreak, including the establishment of emergency guidelines, are needed.

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Effect of intraventricular infusion of anti-prion protein monoclonal antibodies on disease progression in prion-infected mice

Chang-Hyun Song,[1] Hidefumi Furuoka,[2] Chan-Lan Kim,[1]† Michiko Ogino,[1]‡ Akio Suzuki,[1] Rie Hasebe[1] and Motohiro Horiuchi[1]

Correspondence
Motohiro Horiuchi
horiuchi@vetmed.hokudai.ac.jp

[1]Laboratory of Prion Diseases, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan

[2]Department of Pathobiological Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080-8555, Japan

It is well known that anti-prion protein (PrP) monoclonal antibodies (mAbs) inhibit abnormal isoform PrP (PrP^{Sc}) formation in cell culture. Additionally, passive immunization of anti-PrP mAbs protects the animals from prion infection via peripheral challenge when mAbs are administered simultaneously or soon after prion inoculation. Thus, anti-PrP mAbs are candidates for the treatment of prion diseases. However, the effects of mAbs on disease progression in the middle and late stages of the disease remain unclear. This study carried out intraventricular infusion of mAbs into prion-infected mice before and after clinical onset to assess their ability to delay disease progression. A 4-week infusion of anti-PrP mAbs initiated at 120 days post-inoculation (p.i.), which is just after clinical onset, reduced PrP^{Sc} levels to 70–80 % of those found in mice treated with a negative-control mAb. Spongiform changes, microglial activation and astrogliosis in the hippocampus and thalamus appeared milder in mice treated with anti-PrP mAbs than in those treated with a negative-control mAb. Treatment with anti-PrP mAb prolonged the survival of mice infected with Chandler or Obihiro strain when infusion was initiated at 60 days p.i., at which point PrP^{Sc} is detectable in the brain. In contrast, infusion initiated after clinical onset prolonged the survival time by about 8 % only in mice infected with the Chandler strain. Although the effects on survival varied for different prion strains, the anti-PrP mAb could partly prevent disease progression, even after clinical onset, suggesting immunotherapy as a candidate for treatment of prion diseases.

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INTRODUCTION

Prion diseases, such as scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt–Jakob disease (CJD), are fatal neurodegenerative disorders characterized by accumulation of a disease-specific, abnormal isoform of the prion protein (PrP^{Sc}) in the central nervous system (CNS), astrogliosis, neuronal vacuolation and neuronal cell death. The appearance of BSE and variant CJD (vCJD), possibly linked to consumption of food derived from BSE-infected cattle, has increased awareness of prion diseases, but at present there is no effective treatment available for prion diseases. Given that transformation of a normal prion protein (PrP^{C}) to PrP^{Sc} is a central event in the pathogenesis of prion disease, compounds and/or strategies that inhibit PrP^{Sc} formation are of therapeutic interest.

Many compounds or strategies have been reported to inhibit PrP^{Sc} formation, including polyanions, glycosaminoglycans, phosphorothioate oligonucleotides, tetrapyrroles, polyene antibiotics, tricyclic compounds, PrP peptides, dominant-negative PrP, cysteine protease inhibitors, PrP immunization and small interfering RNAs (reviewed by Trevitt & Collinge, 2006). Most of these compounds and treatments antagonize PrP^{Sc} formation in cells persistently infected with prions. However, the anti-prion effects *in vivo* are not always consistent with those observed *in vitro*. Indeed, some of the compounds and treatments protect animals from experimental inoculation with prions or delay the onset of disease when administered before, simultaneously or soon after inoculation with prions via a peripheral route (Ehlers & Diringer, 1984; Farquhar & Dickinson, 1986; Ladogana *et al.*, 1992; Priola

†Present address: Foreign Animal Disease Division, Animal Disease Control Department, National Veterinary Research and Quarantine Service, 480 Anyang-6 dong, Manan-gu, Anyang 430-824, Republic of Korea.

‡Present address: Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

et al., 2000). In addition, only a few compounds, such as amphotericin B, its derivative, MS-8209 and pentosan polysulfate (PPS), can prolong survival of mice infected with prions even when administered in the middle or late stage of prion infection via intracerebral inoculation (Demaimay *et al.*, 1997; Doh-ura *et al.*, 2004). Because intraventricular infusion of PPS at a late stage prolongs the incubation period of the disease in transgenic mice that overexpress PrP (Doh-ura *et al.*, 2004), clinical trials using PPS to treat human prion diseases are moving forward (Todd *et al.*, 2005). The current evidence suggests that PPS treatment of vCJD patients appears to have some beneficial effects, although the specificity of the effects still needs to be evaluated carefully (Rainov *et al.*, 2007).

Anti-PrP antibodies prevent direct interaction between PrP^{C} and PrP^{Sc} in a cell-free conversion reaction (Kaneko *et al.*, 1995; Horiuchi & Caughey, 1999). Subsequent reports have shown that anti-PrP antibodies prevent prion propagation in cells persistently infected with prion (Enari *et al.*, 2001; Peretz *et al.*, 2001; Gilch *et al.*, 2003; Kim *et al.*, 2004b; Perrier *et al.*, 2004; Feraudet *et al.*, 2005). The inhibitory effect of anti-PrP antibodies has also been demonstrated *in vivo*. Transgenic mice expressing monoclonal antibody (mAb) 6H4 were shown to be resistant to prion infection via the intraperitoneal route (Heppner *et al.*, 2001). Moreover, active immunization with recombinant PrP, synthetic PrP peptide or a DNA vaccine has been shown to delay the onset of the disease in mice following peripheral prion infection, although immunization was a prerequisite to obtain the prophylactic effect (Sigurdsson *et al.*, 2002; Schwarz *et al.*, 2003; Goñi *et al.*, 2005; Fernandez-Borges *et al.*, 2006). Passive immunization with anti-PrP antibodies was found to be effective in preventing prion infection via the peripheral route if antibodies were administered shortly after prion inoculation, but was not following intracerebral prion infection or if administered on or after clinical onset following intraperitoneal prion infection (White *et al.*, 2003). These results suggest that anti-PrP antibodies can protect against establishment of prion infection in peripheral tissues and thus may be useful for post-exposure prophylaxis. However, the therapeutic potential of anti-PrP antibodies, including whether or not anti-PrP antibodies antagonize prion propagation in the brain and can inhibit disease progression when applied after clinical onset, remains to be elucidated.

To evaluate the therapeutic effects of anti-PrP antibodies on prion diseases more precisely, we carried out intraventricular infusion of anti-PrP mAbs in mice that had been inoculated intracerebrally with prions. Here, we show that intraventricular infusion of anti-PrP mAbs reduced the level of accumulation of PrP^{Sc} and reduced spongiform changes and gliosis relative to negative controls. Furthermore, we observed prolongation of the incubation time in mice infected with the Chandler strain, even when infusion was initiated at the time of clinical onset of the disease.

METHODS

Antibodies. The following anti-PrP mAbs were used in this study: 106 (IgG2b), 110 (IgG2b), 31C6 (IgG1) and 44B1 (IgG2a). The mAbs 106, 110 and 31C6 recognized linear epitopes consisting of mouse PrP aa 88–90, 83–89 and 143–149, respectively, whereas mAb 44B1 recognized a discontinuous epitope within aa 155–231 (Kim *et al.*, 2004a). Anti-feline parvovirus mAb P2-284 (IgG1) was used as a negative control (Horiuchi *et al.*, 1997). The mAbs were dialysed for 3 days against PBS prior to intraventricular infusion. An Alexa Fluor 488 Protein Labelling kit (Molecular Probes) was used for fluorescent labelling of mAbs.

The following rabbit polyclonal antibodies were used as primary antibodies for immunohistochemistry: B103, which recognizes bovine PrP synthetic peptide aa 103–121 (Horiuchi *et al.*, 1995), anti-glial fibrillary acidic protein (GFAP; Dako) to visualize astrocytes and anti-Iba1 (Wako) to visualize microglia.

Mice and prion strains. All procedures for animal experiments were carried out according to protocols approved by the Institutional Committee for Animal Experiments. Mouse-adapted scrapie strains Obihiro and Chandler were used in this study. For intracerebral inoculation, 4-week-old female ICR mice were purchased from CLEA Japan. Twenty microlitres of 10 % brain homogenate from mice infected with the Obihiro or Chandler strain was injected into the left hemisphere. Twelve-week-old female ICR mice were used to determine the distribution of mAbs and to analyse neuronal toxicity by anti-PrP mAbs.

Intraventricular infusion of mAbs using an osmotic pump. Alzet Mini-Osmotic Pumps, models 2001, 2002 and 2004 (DURECT), were used in this study. Filling of the osmotic pumps with antibody solution was carried out according to the manufacturer's instructions. The pre-filled pumps were then placed in PBS at 37 °C for 24 h. Mice were fitted with a stainless steel cannula supplied with the Alzet Brain Infusion kit (DURECT) and positioned according to stereotaxic coordinates into the left lateral ventricle of the brain (bregma – caudal 1.0 mm, lateral 1.0 mm with a depth of 3 mm below the dura). The osmotic pumps were subsequently implanted subcutaneously into the back and connected to the fitted cannula. All surgical procedures were performed under anaesthesia by intramuscular injection of xylazine (10 mg kg^{-1}) and ketamine (50 mg kg^{-1}). After surgery, cefotaxime (Chugai) was administered subcutaneously (40 mg kg^{-1}) and a gentamicin ointment (Schering-Plough) was pasted on the suture line for 3 days. All mice were housed individually during post-surgery observation periods. Mice that died within a few days of the operation were excluded from the statistical analysis.

Stereotaxic injection of mAbs. Mice were anaesthetized as described above and placed onto a stereotaxic apparatus (Narishige). A linear scalp incision was made and the skull was exposed. Bilateral burr holes were drilled to accommodate stereotaxic placement into the left and right hippocampus (bregma – caudal 2.0 mm, lateral 2.1 mm). Using a Hamilton syringe with a 31-gauge needle, 2 µl mAbs (2 mg ml^{-1}) were injected into the left and right hippocampus, respectively, at a depth of 2 mm below the dura. Injection was carried out over a period of 15 min.

Western blotting. Brains were sagittally hemi-sectioned and homogenized in 10 % (w/v) TMS buffer [50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 5 % glucose]. To detect PrP^{Sc}, 200 µl brain homogenate was mixed with an equal volume of a detergent buffer [8 % Zwittergent 3-14, 1 % Sarkosyl, 100 mM NaCl, 50 mM Tris/HCl (pH 7.5)] and treated with collagenase (0.5 mg ml^{-1}) for 15 min at 37 °C. The samples were then digested with proteinase K (PK; Roche)

at 20 μg ml^{-1} for 30 min at 37 °C. After terminating PK activity by adding Pefabloc (Roche) at 2 mM, samples were treated with 40 μg DNase I ml^{-1} for 5 min. A half volume of a mixture of 2-butanol and methanol (5:1) was added and the PrP^{Sc} was pelleted by centrifugation at 20 000 *g* for 10 min at 20 °C. The resulting pellet was dissolved in 1 × SDS sample buffer [62.5 mM Tris/HCl (pH 6.8), 5 % glycerol, 3 mM EDTA, 4 % β-mercapthoethanol, 0.04 % bromophenol blue, 5 % SDS, 4 M urea] by boiling for 5 min. SDS-PAGE and Western blotting were carried out as described elsewhere (Uryu *et al.*, 2007)

Histopathology and immunohistochemistry. Mouse brains that had been infused with Alexa Fluor 488-conjugated mAbs were frozen in Tissue-Tek OCT compound (Sakura) and cryosections of 16–20 μm were prepared. The sections were dried and fixed with acetone for 10 min. Sections were mounted with Vectashield containing propidium iodine (PI; Vector Laboratories) and examined with a Nikon C1 laser confocal microscope. The presence of infused mAb was also confirmed by direct detection as follows. The sections were reacted with EnVision$^+$ System-labelled polymer conjugated to horseradish peroxidase (HRP) (Dako) for 45 min at 37 °C and positive signals were detected using Simple Stain 3,3′-diaminobenzidine (DAB) solution (Nichirei). This was followed by counterstaining with Mayer's haematoxylin (Wako).

Dissected mouse brains were fixed in 10 % formalin and embedded in paraffin. Sections (4 μm) were deparaffinized, rehydrated and subjected to haematoxylin and eosin (H&E) staining or immunohistochemistry. Antigen retrieval for immunohistochemistry was performed by hydrolytic autoclaving at 135 °C for 20 min for detection of PrP^{Sc} and at 121 °C for 10 min for GFAP and Iba1 (Furuoka *et al.*, 2005). The sections were treated with 3 % H_2O_2 for 5 min, blocked with 10 % normal goat serum for 30 min and then incubated for 45 min at 37 °C with B103 at a dilution of 1 : 100, anti-GFAP at 1 : 5000 or anti-Iba1 at 1 : 100. After washing with PBS, the sections were reacted with EnVision$^+$ System-labelled polymer–HRP for 45 min at 37 °C. The sections were then rinsed and developed with Simple Stain DAB, followed by counterstaining with Mayer's haematoxylin.

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Neuronal cell death was examined using an *In situ* Cell Death Detection kit (Roche). Four micrometer sections of paraffin-embedded brain tissue were deparaffinized, rehydrated and incubated with 10 μg PK ml^{-1} for 10 min at 37 °C. After washing with PBS, the sections were incubated with labelling mixture containing terminal deoxynucleotidyl transferase and digoxigenin-labelled dUTP-conjugated FITC for 60 min at 37 °C. The sections were counterstained with PI and examined with a C1 laser confocal microscope.

RESULTS

Distribution of mAb in brain following intraventricular infusion

We first examined the distribution of mAb infused into the left lateral ventricle of mouse brain. Alexa Fluor 488-conjugated mAb 31C6 (anti-PrP mAb) or P2-284 (negative-control mAb) was infused into the left lateral ventricle and the distribution of mAb was examined at 7, 14, 24 and 34 days after the initiation of infusion. To examine the distribution of the mAbs, brain cryosections at the levels indicated in Fig. 1(a) were prepared. Fig. 1(c) shows the detection of Alexa Fluor 488-conjugated mAb 31C6 in the hippocampus. Fluorescence was detected over the hippocampus (up to 14 days). Although the area of distribution gradually narrowed thereafter, mAbs were still detectable in the hippocampus at 20 days after the termination of infusion (i.e. at 34 days). In contrast, a very low-level fluorescent signal was detected in the hippocampus of a mouse infused with Alexa Fluor 488-conjugated mAb P2-284, even at 7 days after the initiation of infusion. These results suggested that the longer duration of mAb 31C6 in the hippocampus compared with the control mAb was due to binding of mAb 31C6 to PrP^{C}.

Fig. 1(d) summarizes the distribution of the anti-PrP mAb. The mAb was well distributed to areas surrounding the lateral and dorsal third ventricles, hippocampus and thalamus. The mAb was also detected in areas close to the ventral third ventricles. In addition, mAb was detected in regions of the medulla oblongata that face the fourth ventricle and the subarachnoid space, suggesting that the mAb infused into the lateral ventricle was distributed to many parts of brain, presumably via the flow of cerebrospinal fluid. Although the mAb was distributed to parts of the brain parenchyma, distribution of mAb into the cortex and cerebellum appeared to be less efficient. When observing sections under a microscope, we noticed that the mAb-infused hemisphere showed higher fluorescence intensity than that observed in the contralateral side (data not shown), suggesting that the distribution of mAb was not symmetrical. This tendency was confirmed by direct detection of Alexa Fluor 488-conjugated mAb 31C6 (Fig. 1b).

Effects of anti-PrP mAbs on PrP^{Sc} accumulation in the brain

Mice inoculated with Obihiro or Chandler strain reach the terminal stage of the disease at around 150 days post-inoculation (p.i.). Early clinical signs such as ataxia of hind limbs and changes in pelage and posture appear at around 120 days p.i. To evaluate the therapeutic potential of anti-PrP mAbs in a late stage of the disease, infusion of mAbs was started at 120 days p.i. and accumulation of PrP^{Sc} and neurohistopathological lesions were analysed.

Fig. 2(a) shows PrP^{Sc} accumulation in the brains of mice infected with the Obihiro strain at 30 days post-infusion (150 days p.i.). The mean PrP^{Sc} levels in mice treated with mAbs 110, 31C6 and 44B1 were 78, 69 and 77 %, respectively, compared with the control (mAb P2-284; n=2). To determine whether the relative reduction in PrP^{Sc} levels was caused by acceleration of PrP^{Sc} degradation or deceleration of PrP^{Sc} accumulation, we analysed the kinetics of PrP^{Sc} accumulation during the period from 127 to 150 days p.i. (Fig. 2b). There was no difference in PrP^{Sc} levels in mice treated with anti-PrP mAbs compared with those treated with the negative-control mAb at 127 days p.i. (7 days after the initiation of infusion). However, PrP^{Sc} levels increased 2.3-fold in mice treated with the control

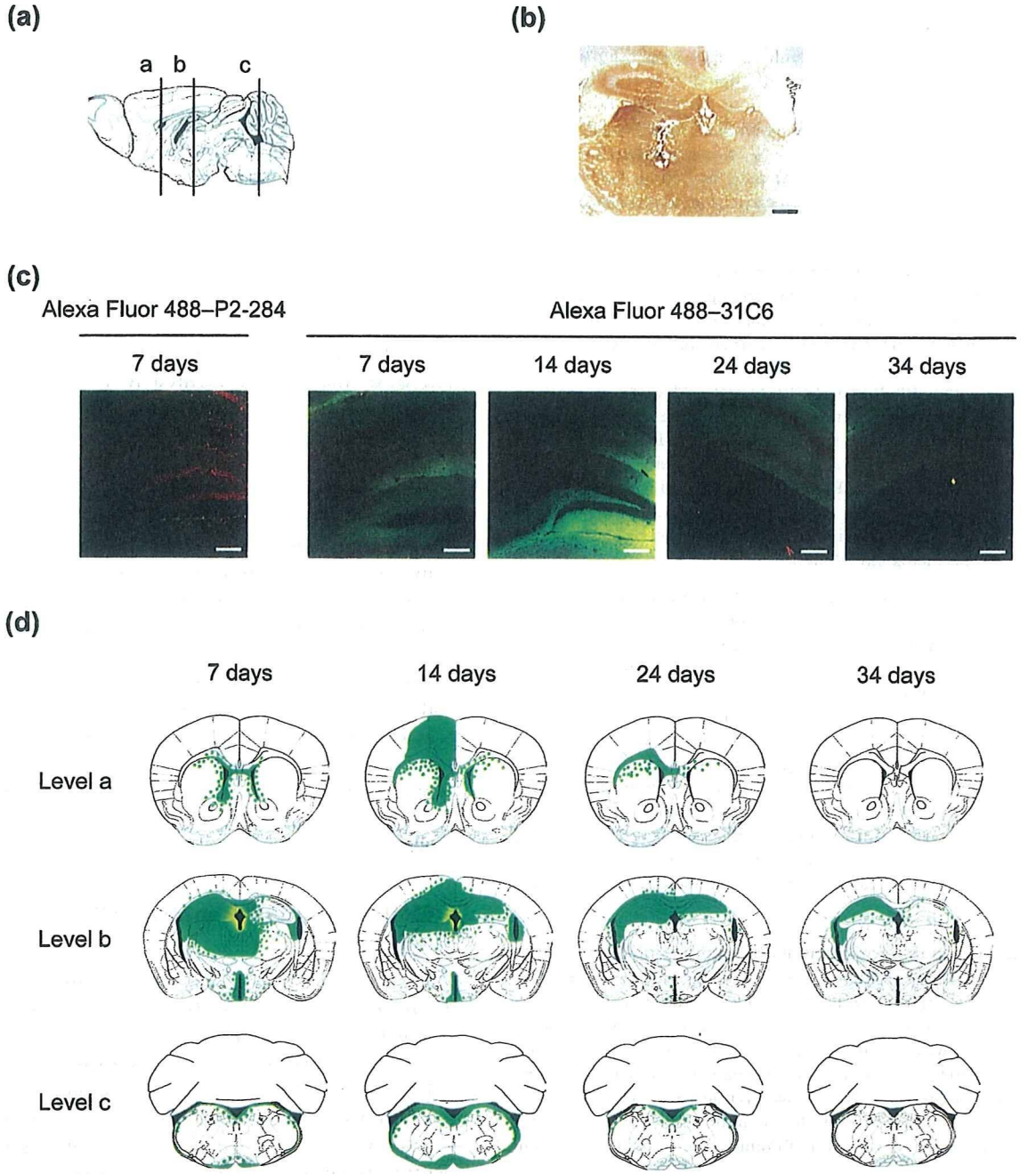

Fig. 1. Distribution of mAbs following intraventricular infusion. Alexa Fluor 488-conjugated mAb 31C6 or P2-284 was infused into the left lateral ventricle using an Alzet Mini-Osmotic Pump model 2002 (mAb concentration 0.5 mg ml^{-1}, pumping rate 0.5 µl h^{-1}, duration 14 days, volume 200 µl). (a) Levels of coronal section examined. Cryosections at the indicated levels were prepared. (b) Detection of mAbs by direct staining. A frozen section at level b was prepared from the brain of a mouse sacrificed at 5 days after starting infusion and the distribution of mAb was visualized by direct staining. Bar, 500 µm. (c) Detection of mAbs in the hippocampus. mAbs conjugated with Alexa Fluor 488 were analysed by laser confocal microscopy. Bars, 200 µm. (d) Distribution of mAb after infusion. The distribution of mAb at 7, 14, 24 and 34 days after starting infusion (green) was superimposed on the images taken from Paxinos & Franklin (2001).

mAb over the period 127–150 days p.i., whereas PrP^{Sc} levels increased only 1.6-, 1.3- and 1.3-fold in mice treated with mAbs 110, 31C6 and 44B1, respectively. These results indicated that anti-PrP mAbs can reduce the rate of PrP^{Sc} accumulation in the brain, even when treatment is initiated at a late stage of the disease.

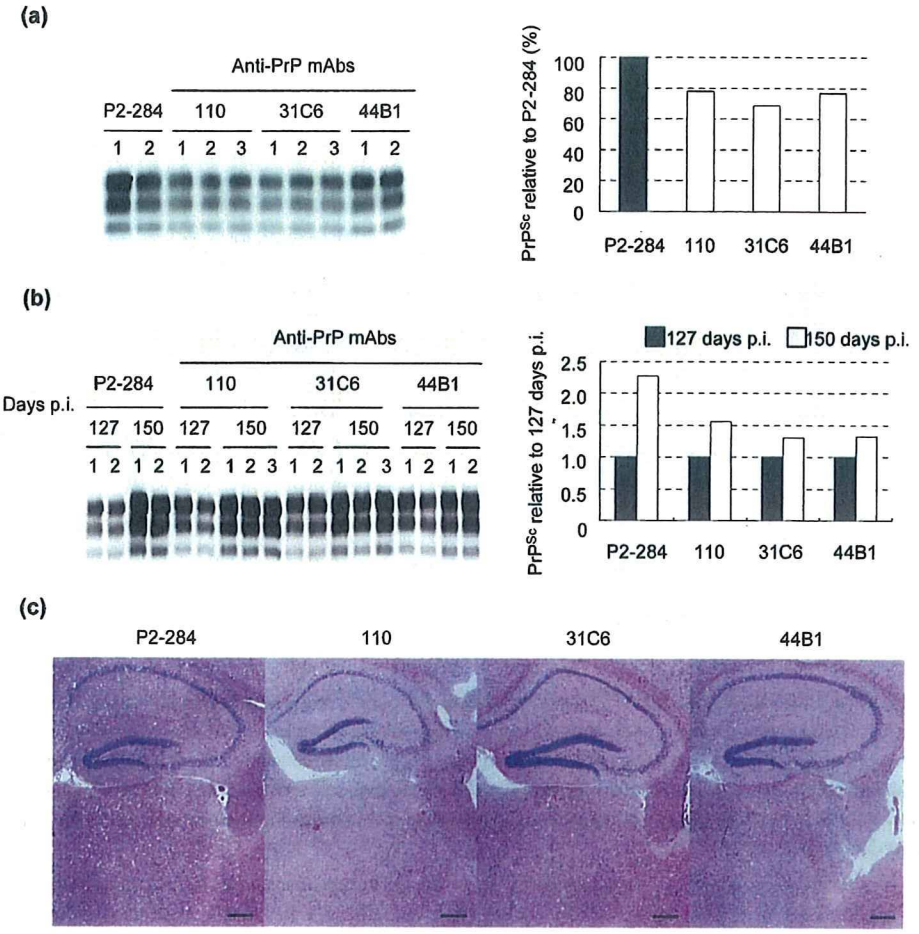

Fig. 2. Effects of anti-PrP mAbs on PrP^{Sc} accumulation and spongiform changes in mice infected with the Obihiro strain. mAbs were infused into the left lateral ventricle of mice inoculated with the Obihiro strain at 120 days p.i. using an Alzet Mini-Osmotic Pump model 2004 (mAb concentration 2 mg ml^{-1}, pumping rate 0.25 µl h^{-1}, duration 28 days, volume 200 µl). Mouse brains were cut sagittally along the midline. The left hemisphere (mAb-infused side) was used for the detection of PrP^{Sc} by Western blotting, whereas the right hemisphere (non-infused side) was fixed with 10 % formalin for paraffin sections. (a) Accumulation of PrP^{Sc} at 150 days p.i. Samples from individual mice (50 µg brain equivalent) were loaded in each lane and the intensities of PrP^{Sc} bands were quantified. The mean intensity for mice treated with the negative-control mAb (P2-284) was designated 100 % and the graph shows relative PrP^{Sc} levels for mice treated with anti-PrP mAbs. (b) Kinetics of PrP^{Sc} accumulation. Mice were sacrificed at 127 and 150 days p.i. and the left hemisphere (mAb-infused side) was used for Western blotting. Samples from individual mice (50 µg brain equivalent) were loaded in each lane and chemiluminescence intensities were quantified. The graph on the right shows the mean level of PrP^{Sc} at 150 days p.i. compared with the level at 127 days p.i. Samples at 150 days p.i. were the same as those in (a). (c) Spongiform changes at 150 days p.i. Paraffin sections were prepared from the contralateral hemisphere of the brain described in (a) and stained with H&E.

Effects of anti-PrP mAbs on neurodegeneration

Next, we investigated the effects of mAbs on neurodegeneration. To do this, the contralateral hemispheres of brains used in Fig. 2(a) (at 150 days p.i.) were examined histopathologically. Although mAbs were more readily detected in the infused side than in the contralateral side (Fig. 1), spongiform changes in the hippocampus and thalamus of mice treated with mAbs 110, 31C6 and 44B1 were nevertheless milder than those treated with the negative-control mAb (Fig. 2c).

Immunohistochemical examination also revealed that anti-PrP mAbs affected the progression of neuropathological lesions in mice infected with the Obihiro strain (Fig. 3). Consistent with the reduction in PrP^{Sc} levels by anti-PrP mAbs (Fig. 2a), PrP^{Sc} deposition in the hippocampus and thalamus of mice infused with mAbs 110 and 31C6 was milder than in the negative control. In addition, astrogliosis (as evaluated by GFAP staining) appeared to be reduced in mice treated with anti-PrP mAbs compared with the negative control. Microglial activation in the

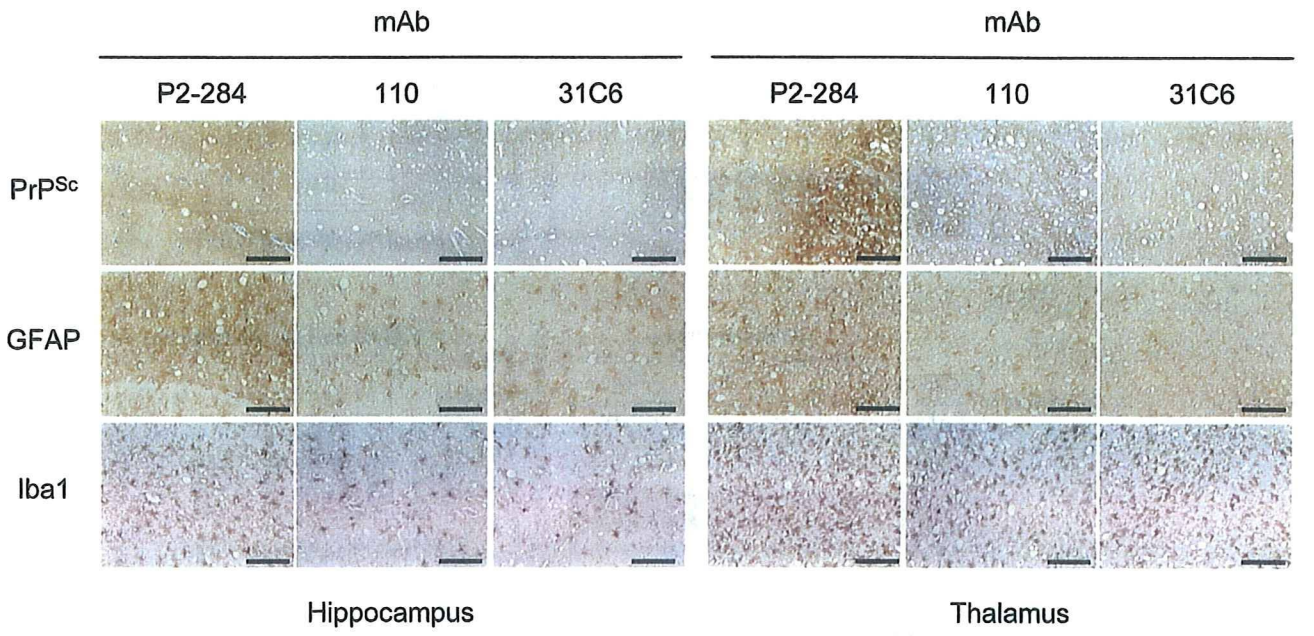

Fig. 3. Effects of anti-PrP mAbs on PrP^{Sc} accumulation and gliosis in mice infected with the Obihiro strain. Intraventricular infusion of mAbs was carried out as described in Fig. 2 and brains collected at 150 days p.i. were used for analysis. The mice shown in this figure belonged to an independent experimental group (i.e. different from the experimental group shown in Fig. 2). Paraffin-embedded sections were stained with B103 antibodies to detect PrP^{Sc}, anti-GFAP antibodies to detect astrocytes and anti-Iba1 antibodies to detect microglia. Images of the hippocampus and thalamus are indicated. All images represent the mAb-infused side. Bars, 200 μm.

hippocampus (as detected with anti-Iba1 antibodies) was also reduced in the presence of anti-PrP mAbs; in contrast, the effect was marginal in the thalamus.

To investigate the effects of anti-PrP mAbs on different prion strains, we carried out the same experiment using mice infected with the Chandler strain. Similar to what was observed for mice infected with the Obihiro strain, in mice infected with the Chandler strain, mAb 31C6 reduced spongiform changes and PrP^{Sc} deposition in the hippocampus and thalamus compared with the negative-control mAb (Fig. 4). However, the effect of anti-PrP mAb on gliosis appeared to differ for the two different prion strains. Anti-PrP mAbs apparently reduced astrogliosis in the hippocampus and thalamus of mice infected with the Obihiro strain (Fig. 3), but only a slight reduction in astrogliosis was observed for mice infected with the Chandler strain (Fig. 4). Moreover, although microglial activation in the thalamus of mice infected with the Obihiro strain was slightly reduced by treatment with anti-PrP mAbs, it was obviously reduced relative to controls by treatment with mAb 31C6 in mice infected with the Chandler strain (Fig. 4). However, microglial activation as a whole appeared to be moderate in mice infected with the Chandler strain compared with the Obihiro strain; thus, the difference observed could be due to a difference in the level of activation of the microglia between mice infected with the two prion strains.

Prolongation of survival time

To determine whether treatment with anti-PrP mAbs can prolong survival of prion-infected mice when administered at different stages in progression of the disease, infusion was started at a middle stage of infection (60 or 90 days p.i.) and after clinical onset (120 days p.i.). In mice infected with the Obihiro strain, infusion of mAb 31C6 initiated at 60 days p.i. prolonged survival by about 11 days compared with the negative control; however, no effect was observed when infusions were initiated at 90 or 120 days p.i. (Fig. 5 and Table 1). In contrast, for mice infected with the Chandler strain, prolongation of survival was observed in all three groups: infusion initiated at 60, 90 or 120 days p.i. prolonged survival by approximately 10, 13 or 12 days, respectively. Brain sections (H&E stained) of all mice were examined for possible causes of death other than prion disease. Of 96 mice tested, two had severe abscesses around the infused area and thus were excluded from the experimental group.

Changes in body weight were consistent with prolonged survival times. For experimental groups in which survival was prolonged by infusion with mAb 31C6, the decrease in body weight observed in control groups was delayed by about 1 or 2 weeks (Fig. 6). In contrast, no difference was observed for mice infected with the Obihiro strain when mAb infusion was started at 90 or 120 days p.i.

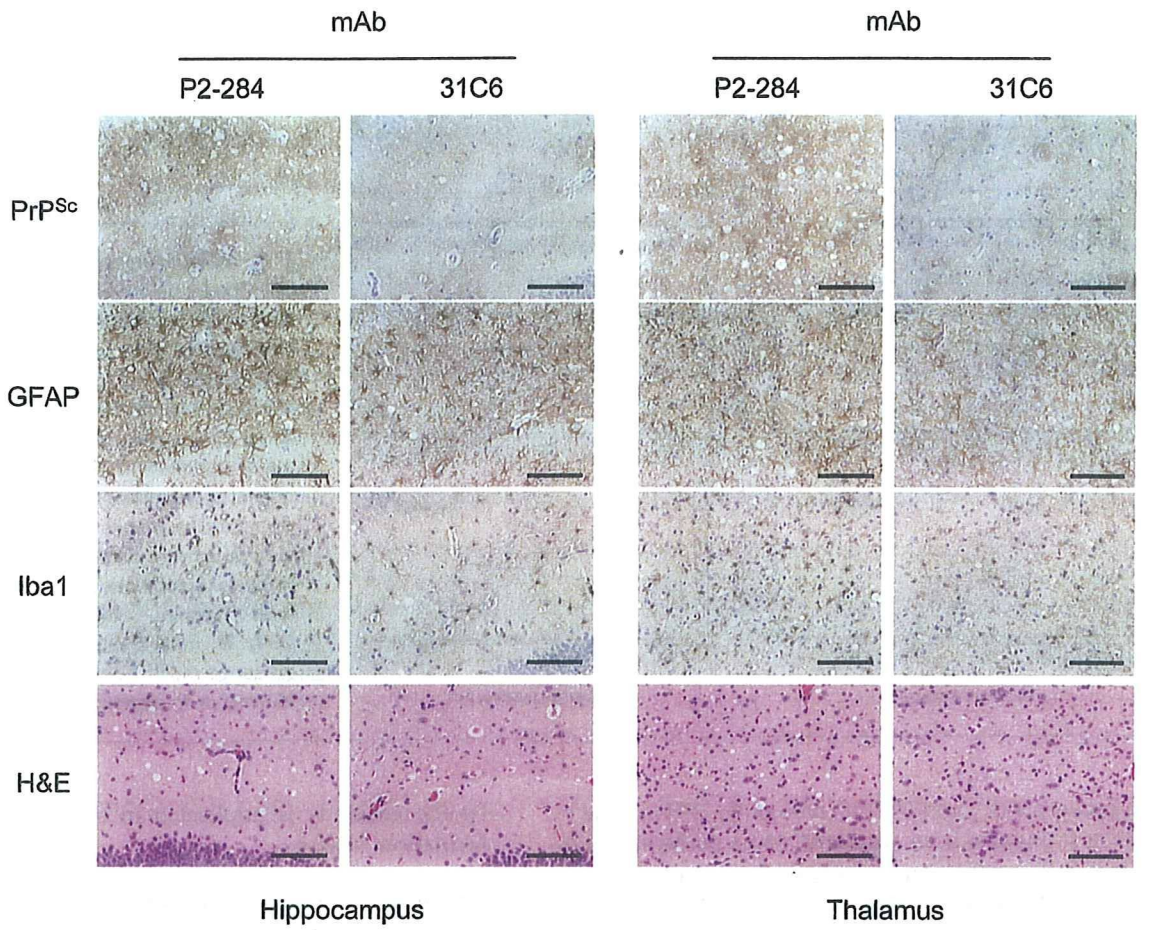

Fig. 4. Effects of anti-PrP mAbs on neuropathological changes in mice infected with the Chandler strain. Intraventricular infusion of mAbs was carried out as described in Fig. 2 using mice inoculated with the Chandler strain and brains collected at 150 days p.i. were used for analysis. The hippocampus and thalamus from mAb-infused hemispheres are shown. Bars, 100 μm.

Neuronal toxicity of mAbs

As it has been reported that an anti-PrP mAb recognizing aa 95–105 of murine PrP can induce apoptosis in hippocampal neurons (Solforosi *et al.*, 2004), we assessed the neurotoxicity of the mAbs used in this study. First, mAbs were infused into the lateral ventricle for 7 days using an Alzet Mini-Osmotic Pump model 2001 (mAb concentration 1 mg ml^{-1}, pumping rate 1 μl h^{-1}, duration 7 days, volume 200 μl); however, no difference was observed between mice treated with anti-PrP mAbs and those treated with the negative-control mAb (data not shown). To assess neurotoxicity directly, we next stereotaxically injected anti-PrP mAbs and the control mAb into the left and right hippocampus, respectively. The distribution of mAb in the hippocampus was confirmed by injecting Alexa Fluor 488-conjugated mAb 31C6 (Fig. 7a). Although mAb was well-distributed throughout the entire hippocampus on the injected side, TUNEL-positive cells were only detected in a limited area of the pyramidal layer and this was observed even in the right side (the side injected with the control mAb P2-284). Indeed, the TUNEL-positive cells were close to the injection site, suggesting that the TUNEL-positive cells resulted from the trauma of mAb injection. It was interesting that mAbs 106 and 110 recognizing the region adjacent to aa 95−105 did not induce apparent neuronal death.

DISCUSSION

In this study, we investigated the effects of anti-PrP mAbs on progression of prion disease, focusing on treatment during late stages of infection. We showed that anti-PrP mAbs antagonized PrP^{Sc} formation in the brain when intraventricular administration was initiated at the time of clinical onset (Figs 2 and 3). The effect of anti-PrP mAbs appeared to be mainly due to deceleration of PrP^{Sc} formation rather than active degradation of PrP^{Sc}. Several reports have suggested that binding of anti-PrP antibodies to the first α-helical domain of PrP^{C} (aa 143–155), which is proposed to be important for the PrP^{C}–PrP^{Sc} interaction (Morrissey & Shakhnovich, 1999; Speare *et al.*, 2003), prevents PrP^{Sc} formation by inhibiting the direct inter-

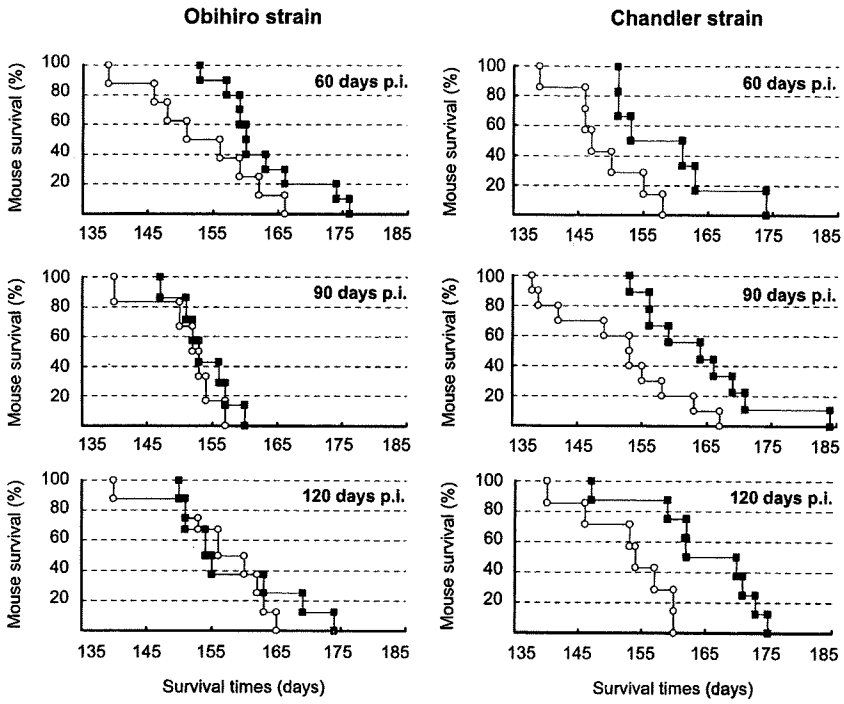

Fig. 5. Intraventricular infusion of anti-PrP mAb prolongs survival. Intraventricular infusion of mAbs into mice inoculated with the Obihiro or Chandler strain was initiated at 60, 90 or 120 days p.i. using an Alzet Mini-Osmotic Pump model 2002 (antibody concentration 2 mg ml^{-1}, pumping rate 0.5 µl h^{-1}, duration 14 days, volume 200 µl). Mice were observed until they reached the terminal stage of the disease. The graphs show survival curves, and survival times (means ± SD) in days are shown in Table 1, together with the numbers of mice in each group. ○, Mice treated with mAb P2-284; ■, mice treated with mAb 31C6. The presence of PrP^{Sc} was confirmed by Western blotting in all experimental-group mice.

action between PrP^{C} and PrP^{Sc} (Enari *et al.*, 2001; Peretz *et al.*, 2001). It has also been suggested that a perturbation of the usual PrP^{C} trafficking by binding of the antibody to PrP^{C} on the cell surface, e.g. sequestration of PrP^{C} on the cell membrane, may be one of the mechanisms of inhibition (Kim *et al.*, 2004b; Feraudet *et al.*, 2005). Consistent with our previous observations (Kim *et al.*, 2004b), mAbs directed against the C-terminal domain (mAb 44B1) and the octapeptide repeat in the N-terminal region (mAb 110), as well as one directed against the first α-helix (mAb 31C6), antagonized PrP^{Sc} formation in the mouse brain (Fig. 2). Indeed, anti-PrP mAb infused into the lateral ventricle was still detectable in the hippocampus at 20 days after the termination of infusion, whereas only a low level of the negative-control mAb was detected in the same region, even during infusion (Fig. 1). These results suggest that the antibody–PrP^{C} complex remains in the brain parenchyma and therefore that sequestration of PrP^{C} by the antibody is implicated in the inhibition of PrP^{Sc} formation *in vivo*.

Intraventricular infusion of anti-PrP mAbs at a late stage of infection (initiated at 120 days p.i.) reduced levels not only of PrP^{Sc} accumulation but also of microglial activation, astrogliosis and spongiform changes. Comparison of neurohistopathological changes observed at 127 versus 150 days p.i. in mice treated with anti-PrP mAbs and the negative-control mAb revealed an apparent reduction in gliosis observed at 150 days p.i., which may be due to a slowdown in the progression of gliosis (data not shown). Although the severity of microglial activation and astrogliosis differed in animals infected with the two different prion strains, the reduction observed was in accordance with the mAb distribution, and the levels of microglial activation and astrogliosis in the hippocampus and thalamus of mice infused with anti-PrP mAbs appeared

Table 1. Effect of intraventricular infusion of anti-PrP mAbs on survival of mice infected with the Obihiro or Chandler strain

Initiation of mAb infusion (days p.i.)	Survival time of mice [mean ± SD (days)]			
	Obihiro strain		Chandler strain	
	P2-284 (*n*)	31C6 (*n*)	P2-284 (*n*)	31C6 (*n*)
60	153.4 ± 9.0 (8)	163.5 ± 8.1* (10)	150.7 ± 10.2 (7)	161.0 ± 8.3* (6)
90	152.8 ± 4.3 (6)	153.3 ± 3.5 (7)	151.7 ± 9.8 (10)	165.0 ± 10.9* (9)
120	154.4 ± 9.4 (8)	157.4 ± 7.0 (8)	152.9 ± 7.4 (7)	164.9 ± 9.3* (8)

*Statistically significant difference based on Student's *t*-test ($P<0.05$).