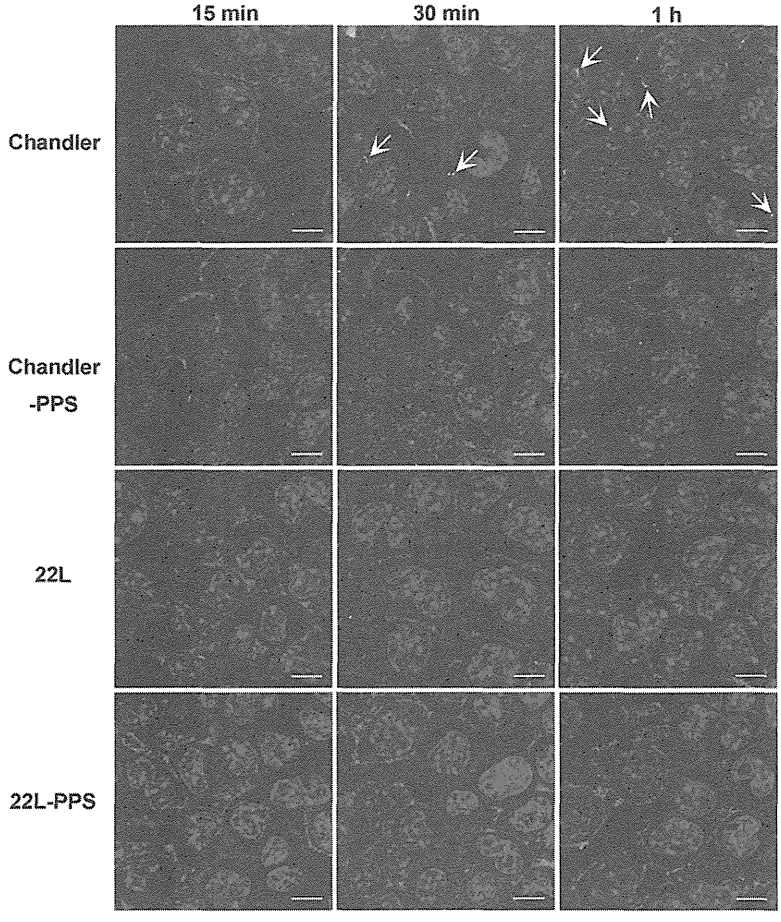

Fig. 3. Localization of PrP and C1q in scrapie-infected N2a cells treated with NMS. The cells were treated with NMS for 15, 30 min or 1 h at 37 °C and fixed with 4% PFA 4% sucrose for double immunostaining of PrP (red) and C1q (green). The nuclei were counterstained with DAPI (blue). Bars show 10 μm. Arrows show merged signals.

possibility is that C3 may bind PrP only in the absence of C1q because of the former's lower affinity to PrP/PrP^{Sc}. Extensive additional experiments would be required to discriminate between these possibilities.

Annexin V has a high affinity for phosphatidylserine (Koopman et al., 1994), which is exposed from the inner layer to the outer layer of the plasma membrane in the early stages of cell death by apoptosis and necrosis (Fadok et al., 1992). In the current study, we found that infected N2a cells were stained with Annexin V after NMS treatment. However, the cells had not progressed to either full-blown apoptosis or necrosis because less than 10% of the cells were stained with PI at 24 h after treatment. In addition, the cells were negative for cleaved caspase-3 at 24 h (data not shown). A similar phenomenon has been reported when human B cells are treated with C3 (Lóbner et al., 2009); i.e., the cells were positive for Annexin V, but were negative for cleaved caspase-3. Segmentation of nuclei was not observed. Therefore, our data suggest that complement factors induced translocation of phosphatidylserine, without cell death. The other factors may be required for cell death in prion infections.

The relatively strong deposition of PrP^{Sc} in the thalami of Chandler- and 22L-infected mice at the preclinical 90 dpi time point seems likely to account for the mild vacuolation and deposition of either C1q or C3, respectively, in this region. Although we do not have any evidence that these complement factors were activated, there is a possibility that complement activation may occur relatively strongly in the thalamus at this time point, and be involved in neuropathogenesis. However, the functional/clinical consequences on the thalamic lesions in particular are not clear because by the onset of clinical signs, the accumulation of PrP^{Sc}, complement factors, gliosis and vacuolation is more evenly distributed throughout the brain.

The immunohistochemistry data showed widespread distribution of C1q both in Chandler- and 22L-infected mouse brains, although the dorsal part of thalamus in 22L-infected mice lacked C1q immunoreactivity. We suspect that signals stimulating C1q synthesis may not be different between Chandler and 22L infection, but that activation of C1q might be limited in 22L infections. Indeed, C1q in normal mouse serum was not involved in inducing Annexin V positivity in 22L-infected N2a cells. With respect to the immunohistochemistry data, we think that reactivity of complement factors may be different in vivo as well as in N2a cells.

It is also unclear whether complement activation might work to alleviate or worsen disease because of multifunctionality of complement factors in vivo. Nonetheless, we suggest two possibilities for the roles of complement factors in prion infections from our current data. One possibility is that complement factors facilitate microglial phagocytosis of prion-infected neurons by exposing phosphatidylserine on the cell surface. Although phosphatidylserine itself works as an "eat-me" signal and promotes phagocytosis (Marguet et al., 1999), it has been reported that binding of C1q and C3b on the cell surface also facilitates phagocytosis. Because phosphatidylserine has been known as a C1q binding molecule (Païdassi et al., 2008), exposure of phosphatidylserine may result in further deposition of C1q, which in turn may accelerate phagocytosis of the target cells. Another possibility for the roles of complement factors is to cause degeneration of prion-infected neurons. Bordin and Whitfield (2003) showed that

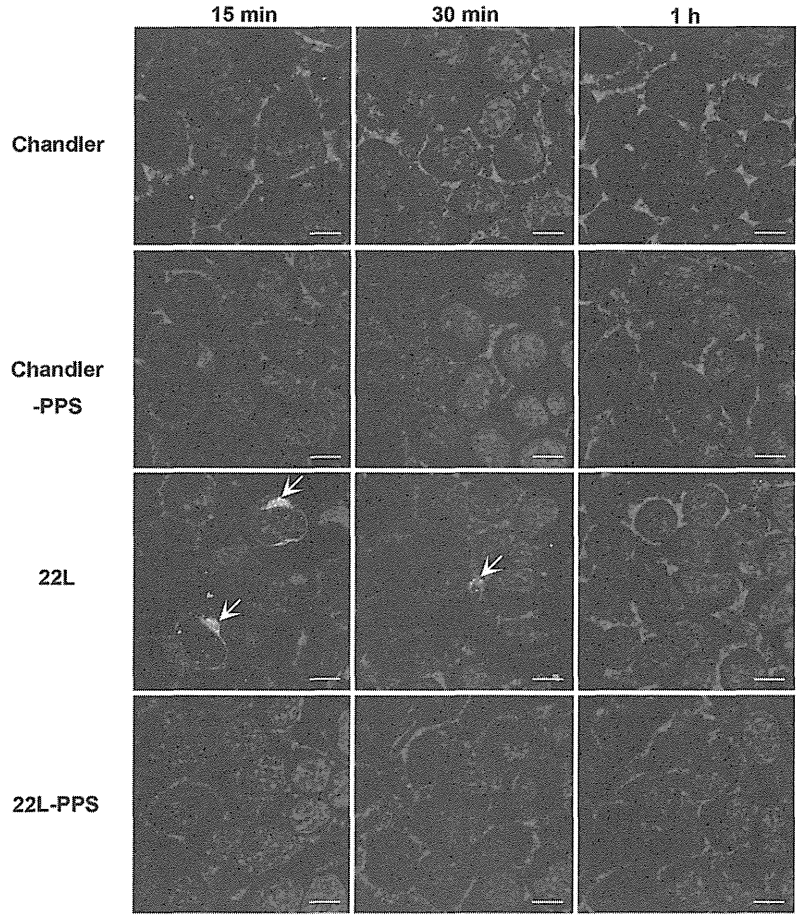

Fig. 4. Localization of PrP and C3 in scrapie-infected N2a cells treated with NMS. The cells were treated with NMS for 15, 30 min, or 1 h and fixed with 4% PFA 4% sucrose in PBS for double immunostaining of PrP (red) and C3 (green). The nuclei were counterstained with DAPI (blue). Bars show 10 μm. Arrows show merged signals.

C1q induced apoptosis in human fibroblasts. In addition, it has been reported that C1q is involved in removal of excess synapses in development (Stevens et al., 2007). C3 is reported to induce translocation of phosphatidylserine from the inner to the outer of the plasma membrane in human B cells (Lóbner et al., 2009). MAC is composed of C5b, C6, C7, C8 and multiple C9 (C5b-9), which form transmembrane channels on the plasma membrane resulting in lysis by fluid influx into the cells. When the number of the C5b-9 molecules on the target membrane is limited, cell lysis does not occur. However, the C5b-9 molecules in sublytic conditions have a pro-apoptotic effect by mediating cellular signaling pathways (Hughes et al., 2000). MAC formed on the Chandler-infected N2a cells could have been sub-lytic in our experiments because cell lysis was not observed. However, some previous studies have reported that complement factors C1q and C3 have anti-apoptotic and neuroprotective effects as well (Benoit and Tenner, 2011; Dashiell et al., 2000; Rus et al., 1996). Interestingly, Erlich et al. (2010) suggested that C1q binds small oligomers derived from murine recombinant PrP and inhibits cytotoxic effects of PrP oligomers. It is possible that complement factors have both neurotoxic and neuroprotective effects and that the role of the complement factors may be different depending on the stage of the disease.

In conclusion, our data provide evidence that the reaction of complement factors varies with the prion strains and that complement reactions can induce the translocation of phosphatidylserine in the membrane of prion-infected N2a cells. The roles of complement factors in prion infection might be further elucidated in the future using ex vivo systems such as slice cultures and mixed cultures of neurons and glial cells.

Materials and methods

Antibodies

Anti-complement C1q, C3 and C9 goat polyclonal antibodies were purchased from Quidal Corporation. Anti-MAC rabbit polyclonal antibodies were purchased from Calbiochem. Anti-PrP mouse monoclonal antibody 6D11 and human monoclonal antibody D13 were purchased from Covance and InPro Biotechnology, respectively. Biotinylated anti-human IgG was purchased from Jackson ImmunoResearch. All Alexa-labeled secondary antibodies were purchased from Invitrogen.

Preparation of mouse serum

NMS was prepared from 6 to 8 weeks old RML mice. Blood was collected from the heart under inhalation anesthesia with isoflulane and coagulated at 4 °C for 6 h. NMS was incubated at 56 °C for 30 min for preparation of H-NMS. Rocky Mountain Laboratories is an AALAC-accredited facility, and all animal procedures were approved by the institution's Animal Use and Care Committee.

Treatment of N2a cells with NMS, HS-NMS and FBS

N2a cells persistently infected with Chandler (Race et al., 1998) or 22L scrapie strains (Kocisko et al., 2003) were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Opti-MEM supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin

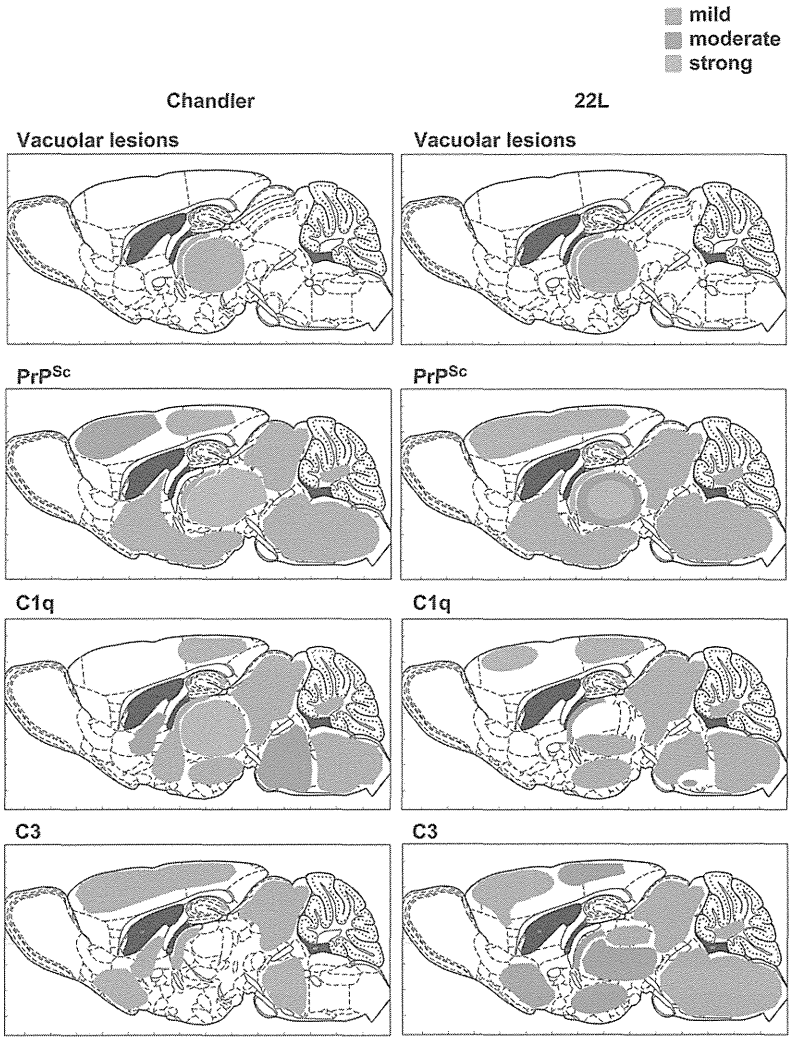

Fig. 5. Summary of distribution of vacuolation, PrP^{Sc}, C1q and C3 of Chandler- or 22L-infected mouse at 90 dpi. The colors indicate the severity of vacuolation or relative intensity of PrP^{Sc}, C1q or C3 deposition. Blue, mild; Green, moderate; Orange, strong; Graphics of the sagittal section of mouse brain are from "The Mouse Brain 2nd edition" (Academic Press). LV: lateral ventricle.

(Invitrogen). For the negative controls, the infected cells were treated with PPS to cure their prion infection. After 5 passages with PPS, PrP^{Sc} was not detected by Western Blotting (data not shown). The cells were seeded on 24 well plates at approximately 10% (for Chandler) or 5% confluence (for 22L) and were cultured for 2 days. Then the cells were cultured with media supplemented with 10% NMS, H-NMS or FBS for 6, 12, 24 and 48 h. To assess inhibitory effects of anti-complement antibodies on the cellular exposure of phosphatidylserine, NMS was treated with anti-C1q, C3 and/or C9 goat polyclonal antibodies at 150 μg/ml of total protein for each antibody. For controls, NMS was treated with NGS or anti-mouse IgG goat polyclonal antibodies at 150 and 450 μg/ml of total protein. Exposure of phosphatidylserine on the outer side of the plasma membrane was detected by Annexin V staining (Invitrogen) according to the manufacturer's instructions. Three areas of each well were randomly chosen and Annexin V positive cells were counted. Proportions of Annexin V positive cells were calculated by dividing by the total number of cells. Three independent experiments were performed. Cell death was detected by PI uptake. The cells were incubated with PI (Invitrogen) at 5 μg/ml for 20 min at 37 °C and observed by fluorescent microscopy (Nikon).

Immunocytochemistry

For MAC detection, the cells were seeded on glass-bottomed Lab-Tek 8 well chamber slides (Nalge Nunc International). At 6, 12 or 24 h after treatment with NMS, the cells were fixed with cold methanol for 15 min, dried and blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline with 0.01% Tween 20. The cells were incubated with anti-MAC antibodies at a 1:100 dilution at 4 °C overnight, followed by incubation with Alexa568-anti rabbit goat polyclonal antibodies at a 1:800 dilution for 1 h at room temperature. The cells were counterstained with 4′6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. For double staining of PrP and C1q/C3, the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min and blocked with 2% BSA for 15 min at room temperature. For the primary antibodies, the cells were incubated with anti-PrP (6D11) mouse monoclonal antibody at a 1:1000 dilution and anti-C1q or C3 goat polyclonal antibodies at a 1:100 dilution. For the secondary antibodies, Alexa488-labeled anti-goat IgG polyclonal antibodies and Alexa 568-labeled anti-mouse IgG polyclonal antibodies were used at a 1:800 dilution for each. The samples were observed with an LSM 510 microscope (Carl Zeiss Inc).

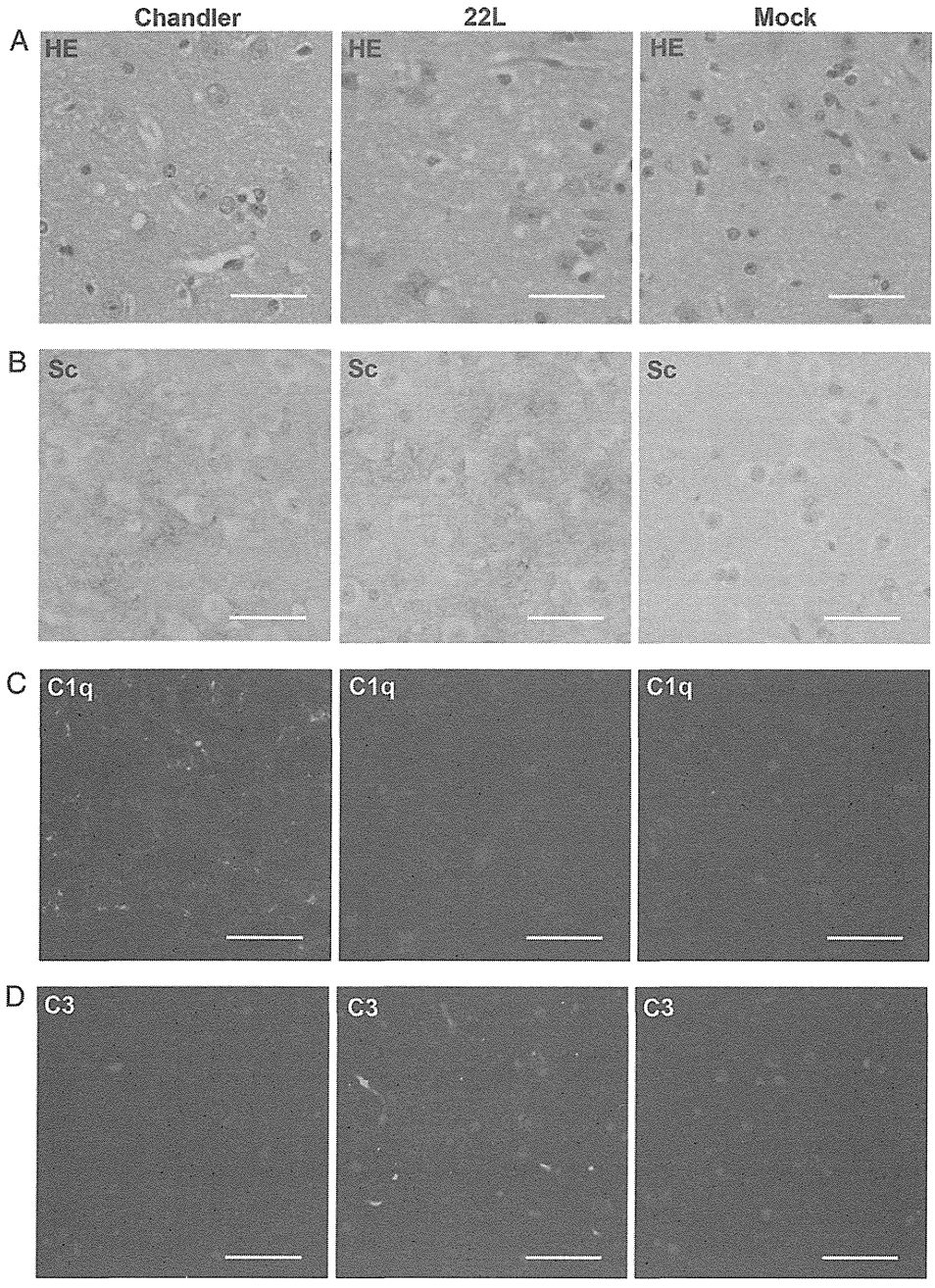

Fig. 6. Histopathology of the dorsal part of thalamus at 90 dpi. The slides from Chandler-, 22L- or mock-infected mouse brains were subjected to HE staining (A) and immunohistochemistry for the detection of PrP^{Sc} (B), C1q (C) and C3 (D). Green, C1q or C3. Blue, DAPI. Bars show 50 μm.

Histological analysis

C57BL/6 mice (8–10 weeks old) were infected intracerebrally with 0.5% Chandler-, 22L-, or normal mouse brain homogenates under inhalation anesthesia with isoflulane. At 90, 133 and 166 dpi, 4 mice for each strain were euthanized and the brains were harvested. The samples were fixed with 10% phosphate-buffered formalin, dehydrated and embedded in paraffin. The paraffin blocks were cut at 4 μm and subjected to HE staining and immunohistochemistry for PrP^{Sc}, C1q and C3. PrP^{Sc} staining was performed using a Ventana automated Discovery XT stainer. For antigen retrieval, the slides were incubated in CC1 buffer (Ventana) containing Tris–borate–EDTA pH 8.0 for 180 min at 95 °C. Then the cells were incubated with anti mouse PrP human antibody D13 at a dilution of 1:500 at 4 °C for 16 h, followed by a biotinylated anti-human IgG at 1:500 and avidin-horseradish peroxidase. Then the slides were reacted with diaminobenzidine (Ventana) as chromogen and observed with an Olympus BX51 microscope. For C1q and C3 detection, the slides were pretreated with 0.03% proteinase K at 37 °C for 30 min, incubated with anti-C1q or C3 antibodies at 4 °C overnight. Then the slides were incubated with Alexa488-labeled anti goat IgG for secondary antibody and counterstained with DAPI.

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REVIEW *Public Health*

Alternative BSE Risk Assessment Methodology for Beef and Beef Offal Imported into Japan

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ABSTRACT. The Food Safety Commission (FSC) of Japan, established in July 2003, has its own initiative to conduct risk assessments on food stuffs known as "self-tasking assessment". Within this framework, the FSC decided to conduct a risk assessment of beef and beef offal imported into Japan from countries with no previous BSE reports; thus, a methodology was formed to suit to this purpose. This methodology was partly based on the previous assessments of Japanese domestic beef and beef imported from U.S.A./Canada, but some modifications were made. Other organizations' assessment methods, such as those used for BSE status assessment in live cattle by the OIE and EFSA's GBR, were also consulted. In this review, the authors introduce this alternative methodology, which reflects (1) the risk of live cattle in the assessed country including temporal risks of BSE invasion and domestic propagation, with the assessment results verified by surveillance data, and (2) the risk of beef and beef offal consisting of cumulative BSE risk by types of slaughtering and meat production processes implemented and the status of mechanically recovered meat production. Other possible influencing factors such as atypical BSE cases were also reviewed. The key characteristic of the current assessment is a combination of the time-sequential risk level of live cattle and qualitative risk level of meat production at present in an assessed country.

KEY WORDS: beef, BSE, importation, prion diseases, risk assessment.

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More than 20 years have passed since BSE was officially recognized in the U.K. Now, there is prominent evidence showing the efficacy of a real feed ban and the abolishment of using meat and bone meal (MBM) derived from mammals in feeds for mammals. The total number of BSE-positive cases in the world last year was less than that of one day when the BSE outbreak was at its peak in the U.K. from 1992 through 1993. However, the U.K. continued to spread the sources of BSE pathogens, such as live cattle and animal feeds, to two dozen countries, resulting in a cumulative number of more than 220 variant CJD patients in the world [9].

Currently, Japan imports beef and beef offal from the U.S.A. and Canada, two countries that have previously experienced BSE cases and for which the Food Safety Commission (FSC) in Japan has already assessed the BSE risks of their beef and beef offal. Besides these two countries, Japan also imports beef and beef offal from other countries where no BSE cases have been reported so far. However, some of these countries were categorized as Geographical BSE Risk (GBR) category III by the European Food Safety Agency (EFSA). According to EFSA's definition, countries are designated as GBR category III either because they are estimated to have a reasonably high possibility of having BSE cases that have not been detected or because they

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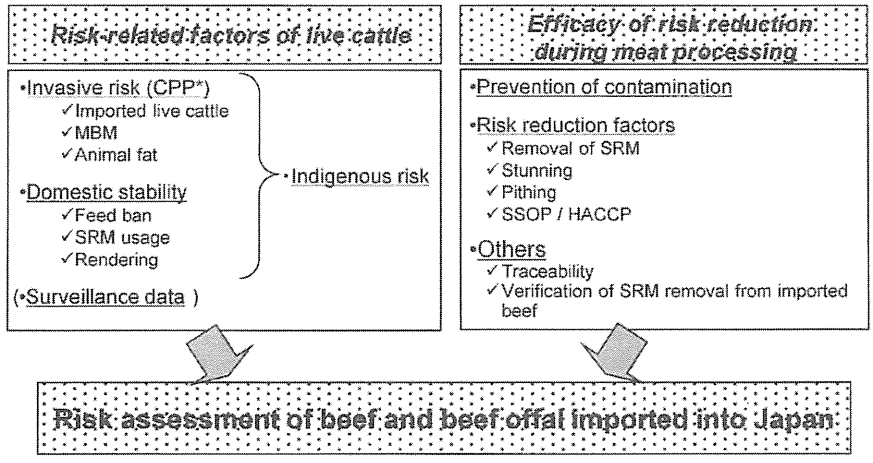


Fig. 1. Schematic presentation of the risk assessment methodology employed by the PEC for the current assessment. Briefly, the risk-related factors in live cattle (shown in the left box) were combined with the risk-reducing efficacy at meat processing facilities (shown in the right box) to gain the total risk assessment targeting beef and beef offal imported into Japan. *: Contamination probability points.

have had a few confirmed cases of BSE. Among exporters to Japan, there are also countries that have simply not been assessed by EFSA's GBR.

Japanese risk managers presently request importers of beef and beef offal from the above countries to submit official health certificates confirming that the cattle are of healthy origin and also ask that they refrain from importing specified risk materials (SRM). Although the health certificates are confirmed at quarantine stations, there are currently no measures to clarify the exclusion of SRM among beef products imported. There is also uncertainty over potential risks of imported beef and beef offal due to insufficient availability of data related to BSE prevalence and anti-BSE countermeasures in the above-mentioned countries.

The FSC in Japan conducts risk assessments at the request of risk managers, or alternatively, it can also conduct assessments on its own initiative, termed "self-tasking assessment". The process of hazard selection for self-tasking assessment is as follows. The Expert Committee for Planning collects information and screens the possible assessment subjects based on the degree of public concern in Japan, based on demands for information collection either due to increasing necessity for developing hazards or based on items that are heavily requested for assessment. Selected subjects are then discussed for potential assessment at the Commission's opinion exchange meetings, and finally, the FSC officially adopts the hazards of choice to be the next subject of self-tasking assessment.

Risk assessment of beef and beef offal imported into Japan was among the most requested items during public meetings and other occasions hosted by the FSC. Behind these requests, there seemed to be public concern over uncertainty about BSE risks in beef and beef products imported from countries other than the U.S.A. and Canada. With this situation, the FSC decided to conduct "risk assessment of beef and beef offal imported into Japan" as its self-tasking assessment.

The current assessment conducted by the Prion Expert Committee (PEC) of the FSC in Japan is based on the following concepts: (1) presently, the worldwide BSE prevalence is in the trend of decline; (2) this risk assessment is essentially different from the rest of the BSE-related risk assessments previously conducted by the FSC, in that the assessed countries are only those that have not previously reported BSE cases; (3) previous risk assessments of beef and beef products from the U.S.A. and Canada were conducted by comparing their risks with that of Japanese beef and beef products so that the assessment was based on the relativity; and (4) it was foreseen to be based on the data submitted by each assessed country on a voluntary basis. Subsequently, assuming that there may be certain limitations concerning data availability and submission, the PEC decided to largely conduct this assessment on a qualitative basis but to strive to make it as quantitative as possible.

It was with this background that the PEC firstly developed an alternative assessment method suited to the current situation and then carried out BSE risk assessment for imported beef and beef offal according to this method. In this review, the authors describe the structure and logic of this assessment method. A sample assessment result is provided at the end of this article to enhance readers' understanding.

PRINCIPLES OF THE CURRENT RISK ASSESSMENT

The methodology for the current risk assessment was developed based on the previously used models for risk assessments of Japanese domestic beef and for US/Canadian beef imported into Japan [5, 6]. OIE's risk assessment criteria for BSE status and the EFSA GBR method were also referred to [8, 11]. The PEC for the current assessment aimed

to deliver the overall conclusion as a science-based comprehensive assessment defined by time periods and based on a combination of the following risk aspects: (1) periodic BSE risk status among the cattle population of a country, which consisted of combined risks of BSE invasion by imported live cattle and MBM, and domestic stability (inversed risk of BSE propagation), of which the latter included implementation of a feed ban and establishment of preventive measures against cross-contamination, etc., and (2) present risks of beef and beef offal processing lines, i.e., risks based on types of slaughtering and meat production processes, etc.(Fig. 1).

The current assessment was conducted on a qualitative basis rather than a quantitative basis because of the limited data regarding BSE risks. In case the data were insufficient, assessment was based on the worst-case scenario. In addition, a few cases of atypical BSE, which is biologically and biochemically different from typical BSE, have been recently found in Europe, Japan and the U.S.A. among other countries. Those cases were distinguished from the classical type of BSE mainly by band patterns of PrP^{Sc} proteins demonstrated by Western blotting. The origin of atypical BSE is still unknown to this date, and information about BSE prion distribution in bovine tissue is scarce [2, 3].

Due to the above-mentioned situation, therefore, the current risk assessment was conducted with the assumption that (1) the first case of BSE occurred in the U.K. for an unknown reason, with BSE agents then being propagated through MBM recycling from BSE-infected cattle, and that (2) BSE infection was spread to other countries by exportation and utilization of BSE-infected live cattle and BSE-contaminated MBM for animal feeds.

PERIODIC BSE RISK STATUS AMONG THE CATTLE POPULATION OF AN ASSESSED COUNTRY

Assessment of invasive BSE risk: For the purpose of analysis in this section, the PEC defined a country in categories III or IV according to the EFSA GBR or a country with at least one BSE-positive case reported among its domestic cattle in the past as a "BSE risk country". Invasive BSE risk was assessed based on their records of live cattle, MBM and animal oil/fat importation from the BSE risk countries defined by this description.

The determined BSE risk countries were divided into the following subgroups by the level of BSE contamination as follows: the U.K., European countries with moderate contamination, European countries with low contamination, the U.S.A., Canada and others (Japan, Mexico, Chile, etc.).

Accordingly, each assessed country exporting beef and beef offal to Japan was requested to submit data regarding imports of live cattle and MBM from the BSE risk countries. Portugal had been categorized as a level IV country by the FESA GBR together with the U.K. and thus should not be grouped with other moderate-risk European countries. Nevertheless, such distinction was not made because no assessed country had a record showing importation from Portugal.

Submitted information was analyzed for possible use of the imported live cattle and MBM for animal feed production in the assessed country. In the case that the records submitted by the assessed country indicated any degree of possibility of live cattle and MBM imports from BSE risk countries having been used for animal feed, the degree of invasive BSE risk in the assessed country was estimated as a sum based on contamination probability points (CPP) of each BSE risk country. The assessment was based on a 5-year period, as this was considered to be the general term for BSE incubation.

Risks of animal oil and fat varied depending on the products' grades such as yellow grease, fancy tallow, etc., but their risks were generally regarded as low compared with that of live cattle or MBM. Thus, the information associated with animal oil/fat and their usages were taken into consideration only when importation of large quantity was recorded from BSE risk countries. Otherwise, the data were used as supplementary information.

Contamination probability points (CPP): All imported live cattle and MBM, in principle, have a potential to be used for animal feed manufacturing, but when a country could provide a feasible explanation for not utilizing any of the imported live cattle or MBM for animal feed, they could be determined as carrying no risk and thus excluded from consideration of risks.

When the track records of the imported live cattle and MBM in the assessed country showed any of the following destinies, they were regarded as adding no risk to the assessed country: (1) imported live cattle were already dead and disposed of by burial or incineration; (2) imported live cattle were still alive at the time of investigation, so they were excluded from potential use for animal feed manufacturing beforehand; and (3) imported live cattle and MBM were recorded to have been re-exported to other countries.

In this assessment, the PEC defined the invasive BSE risk as combined CPPs of imported live cattle and MBM. Its assessment was to be calculated based on the assumption that 1 ton of MBM was equivalent of 1 live bovine animal, as has been stated in the GBR by the Scientific Steering Committee (SSC) and EFSA [10].

The risks of imported live cattle and MBM from the BSE risk countries varied depending on the country and timing of importation. To reflect this variation, this assessment employed CPPs for live cattle and MBM of each BSE risk country. Records showed that the BSE prevalence in the U.K.'s live cattle was 5% at its peak period of 1988–1993; therefore, a CPP of 1 was set as the risk of importing 1 live bovine animal from the U.K. during this period. Thus, the CPPs of the U.K. were set as shown in Table 1 based on the values indicated by the SSC's GBR and years of complete feed ban implementation in the U.K. [10].

European countries except for the U.K. were divided into two categories, namely countries of "moderate contamination" and "low contamination" [11]. The CPPs for live cattle and MBM were set up based on the SSC's GBR and years of complete feed ban implementation in European countries [4, 10] (Table 2). Countries such as France, the Netherlands, Belgium, and Italy were likely countries to have re-exported MBM from the U.K. and thus were given a CPP of 0.1 until

Table 1. Periodic CPPs of live cattle and MBM from the U.K.

Live cattle		MBM (1 ton)	
1987 and years before	0.1		
1988–1993	1	1986–1990	1
1994–1997	0.1	1991–1993	0.1
1998–2005	0.01	1994–2005	0.01
2006 and years after	0.001	2006 and years after	0.001

the U.K. banned exportation of MBM (years of 1986–1996).

In the previous risk assessments done by the PEC of the FSC in Japan concerning U.S.A./Canadian beef imported into Japan, the surveillance-based BSE prevalence of U.S.A. and Canadian cattle were estimated to be 1 case and 5–6 cases per one million cattle in the U.S.A. and Canada, respectively [6]. Accordingly, the CPPs of live cattle and MBM in those two countries were set. The values are given for the periods defined by estimated year of birth among BSE-positive cattle (Table 2).

In the previous risk assessment done by the PEC of the FSC concerning U.S.A./Canadian beef imported into Japan, the surveillance-based BSE prevalence of Japanese cattle were estimated to be 5–6 cases per one million cattle. Birth years of BSE-positive cattle and the year of feed ban implementation were also taken into account to set the following CPPs for live cattle and MBM of Japan [6] (Table 2).

The CPPs for countries with no reported BSE cases could not be set by the above-mentioned BSE prevalence-based method. Since those countries were generally considered to have low BSE risks compared with countries with BSE-positive cases, CPPs were not determined for these countries. In the case that an assessed country imported a large quantity of live cattle and/or MBM from BSE-negative and GBR III countries, the information was taken into consideration as a supplementary factor for the assessment.

Assessment for invasive BSE risk: Based on the principles above, total invasive BSE risk (a sum of the invasion risks from imported live cattle and MBM) was estimated for each assessed country for a period of five years. The assessment was finally given in 5 levels, high, moderate, low, very low and negligible, as shown in Table 3.

Domestic Stability (inversed risk of BSE propagation of a country). Principles of domestic stability assessment: The essential countermeasures against BSE exposure/propagation consisted of (1) implementation of a feed ban, (2) control of SRM use, (3) optimization of rendering conditions and (4) establishment of preventive measures against cross-contamination for feed production.

Previous epidemiological analyses indicated that the most effective measure implemented in Europe was feed ban. Thus, an essential part of BSE exposure/propagation prevention was to abolish feeding of cattle with possibly BSE-contaminated MBM through animal feeds. It is in this context that a feed ban has been implemented in countries as a preventive measure against BSE. At the pragmatic level, the most effective way was to ban recycling of animal proteins regardless of animal types among mammals (ban from mammals to mammals), followed by less but still effective measures such as a ban on protein recycling from mammals to ruminants and then from ruminants to ruminants.

Table 2. CPPs of live cattle and MBM from various countries

European countries with moderate contamination[a]	
1986–2005	0.01
2006 and years after	0.001
European countries with low contamination[b]	
1986–1990	0.001
1991–2005	0.01
2006 and years after	0.001
U.S.A.	
1993–	0.00002
Canada	
1989–	0.0001
Japan	
1992–2006	0.0001
2007 and years after	0.00001

a) France, Netherlands, Belgium, Italy, Ireland, Germany, Spain, Swiss, etc. b) Poland, Denmark, Austria, Czec Republic, Slovenia, etc.

Table 3. Total invasive BSE risk

Levels for risk of invasion	U.K. equivalent (N) [a]
High	100≤N
Moderate	20≤N<100
Low	10≤N<20
Very low	5≤N<10
Negligible	0≤N<5

a) Calculated based on the assumption of 1 ton of MBM equals 1 live bovine animal.

Other measures that were also indicated as important for BSE control in these analyses included exclusion of SRM from rendering materials, optimization of rendering conditions (not less than 133°C for a minimum of 20 min at an absolute pressure of 3 bar), dedication of feed mills to a single species and production line separation.

It has been stated that 99% or more of infectivity in BSE-positive bovine animals is distributed to the bodily regions called specified risk material (SRM; e.g., brain, spinal cord, etc.) [4]. Removal of SRM from rendering materials was considered to be important, and the best way to realize this measure was implementation of a legally-bound feed ban that prohibited the use of SRM and fallen stock in animal feed. Even diversion of SRM use from feed production to human consumption was considered to provide a certain degree of protection against BSE exposure/propagation, when coupled with avoidance of fallen stock use in animal feed.

Rendering under proper conditions could provide an effective reduction in BSE infectivity. For example, heat treatment (126°C for 30 min) of a prion strain (301V strain) after passage using mice resulted in reduction of infectivity by

log1.9 (ID_{50}/g) or log 2.7 (ID_{50}/g). The scientific opinion of the EFSA is that heat treatment of BSE prion under certain conditions (133°C for a minimal of 20 min at bar 3) effectively reduces its infectivity by 1,000 times [12], although the same rendering conditions may not be as effective as indicated above when the subject is a mixture of SRM and bones originating from BSE-infected cattle. Drawn from this data was the suggestion that, although the heat treatment recommended by the OIE appeared to be effective in reducing risks to certain extent, other measures should be combined with this rendering policy to completely prevent BSE exposure/propagation.

A previous study reported that oral administration of 0.1, 0.01 and 0.001 g of BSE-infected cattle brain was capable of transmitting BSE at the rate of 7 in 15 cattle, 1 in 15 cattle and 1 in 15 cattle, respectively [13]. These data were consistent with the European field observation that even a trace amount of animal protein was enough to infect bovine animals through contaminated feeds. Therefore, simply washing of the processing lines in a feed factory was not sufficient to prevent cross-contamination; implementation of more advanced measures such as dedication of feed mills to a certain species of animals and line separation were required.

For assessment of domestic stability, the information submitted from each assessed country was firstly analyzed for the extent of feed ban implementation, and then other aspects such as use of SRM, rendering conditions and preventative measures against cross-contamination were evaluated.

The assessment also focused on the degree of legal obligation bound to each regulation. The data regarding compliance with those preventative regulations were also evaluated whenever the data were available.

Assessment of domestic stability: Based on the assessment principles described above, countries were firstly assessed for their domestic stability by rigidity of feed bans (e.g., abolishing the use of all mammalian products for mammalian feed, mammalian products for ruminant feed or ruminant products for ruminant feed regulations [①–④ in Fig. 2]). Secondly, regulation of SRM usage was considered (◎–○). In the case that SRM was used for feeds, rendering conditions and avoidance of cross-contamination were considered. Risk reduction measures such as rendering at 133°C for 20 min at 3 bars and/or prevention of cross-contamination at feed factories, e.g., isolating production lines or establishing exclusive feed production facilities were also taken into account (○ or △). Finally, the domestic stability of each country was assessed by categorizing them into one of 5 ranks, very stable, stable, middle, unstable and very unstable, based on a certain period defined by significant events such as regulatory modification.

Assessment of overall BSE risk in a cattle population, aided by verification with surveillance: Countries with high BSE propagation risks presumably bear a high risk of domestic BSE exposure/propagation upon entrance of BSE agents into the country. Thus, when the submitted data indicated a high risk of domestic BSE exposure/propagation (namely, combined risks of high invasive BSE risk and unstable domestic stability), this aspect was taken into consideration as an additional factor. In the case that an assessed country was categorized in a high exposure/propagation period at a certain time in the past, that country was assigned to a one-rank higher overall risk group for the next 5 years.

Surveillance was the essential method to scientifically verify the risk assessment output, and so surveillance data obtained from an assessed country was used for the results of the current assessment. The actual verification process was constructed based on the OIE point system (BSurvE method), as no other alternatives were available at that time.

RISK ASSESSMENT OF THE PROCESS OF BEEF AND BEEF OFFAL PRODUCTION

Ensuring the removal of SRM can remarkably reduce the risk of variant Creutzfeldt-Jakob disease (vCJD) in humans. Therefore, this measure is currently at the center of preventive policies regarding protection of human and cattle health from BSE. The current risk assessment for beef and beef offal firstly evaluated the extent of "SRM removal" done by each country, followed by combined assessment of items such as "inspection at slaughterhouses" and "stunning/pithing" to evaluate the risk-reducing efficiency of meat processing lines.

SRM removal: In a BSE-positive bovine animal, 99% or more of its infectivity is attributed to SRM [9]. Thus, exclusion of these materials from the food chain ensures reduction in most of the vCJD-associated risks in humans. To reflect these SRM-related aspects, the current assessment also took into consideration factors such as implementation of SRM removal or any other measures in preventing beef and beef offal from being contaminated by BSE agents. This part of the assessment was ultimately designed to determine whether cross-contamination preventive measures and efficacy verification systems for them had been established in each country.

The definition of SRM adopted in this assessment was from the OIE's SRM definition for a "controlled risk country" based on the following reasons: (1) the currently assessed countries all had no BSE-positive reports; (2) there were, however, some countries that were categorized into GBR III of the EFSA; and (3) the definition and handling of SRM varied among the assessed countries. When the SRM definition of the assessed country was acknowledged to be largely different from that of the OIE, the assessment was conducted in a manner separate from these general principles and handled on a case-by-case manner.

Inspection, stunning and pithing at slaughterhouses: Elimination of high risk cattle such as downer cows is an important protocol in protecting human health from BSE risks, and for this reason, the OIE code requires proper antemortem inspection before slaughtering [9]. However, it is also known that clinical observation for possible symptoms alone is not enough to distinguish BSE-infected cattle from other diseases. Therefore, both the provisions of (1) effective elimination of downer cows at the antemortem inspection and (2) BSE testing at slaughterhouses were evaluated in the current assessment.

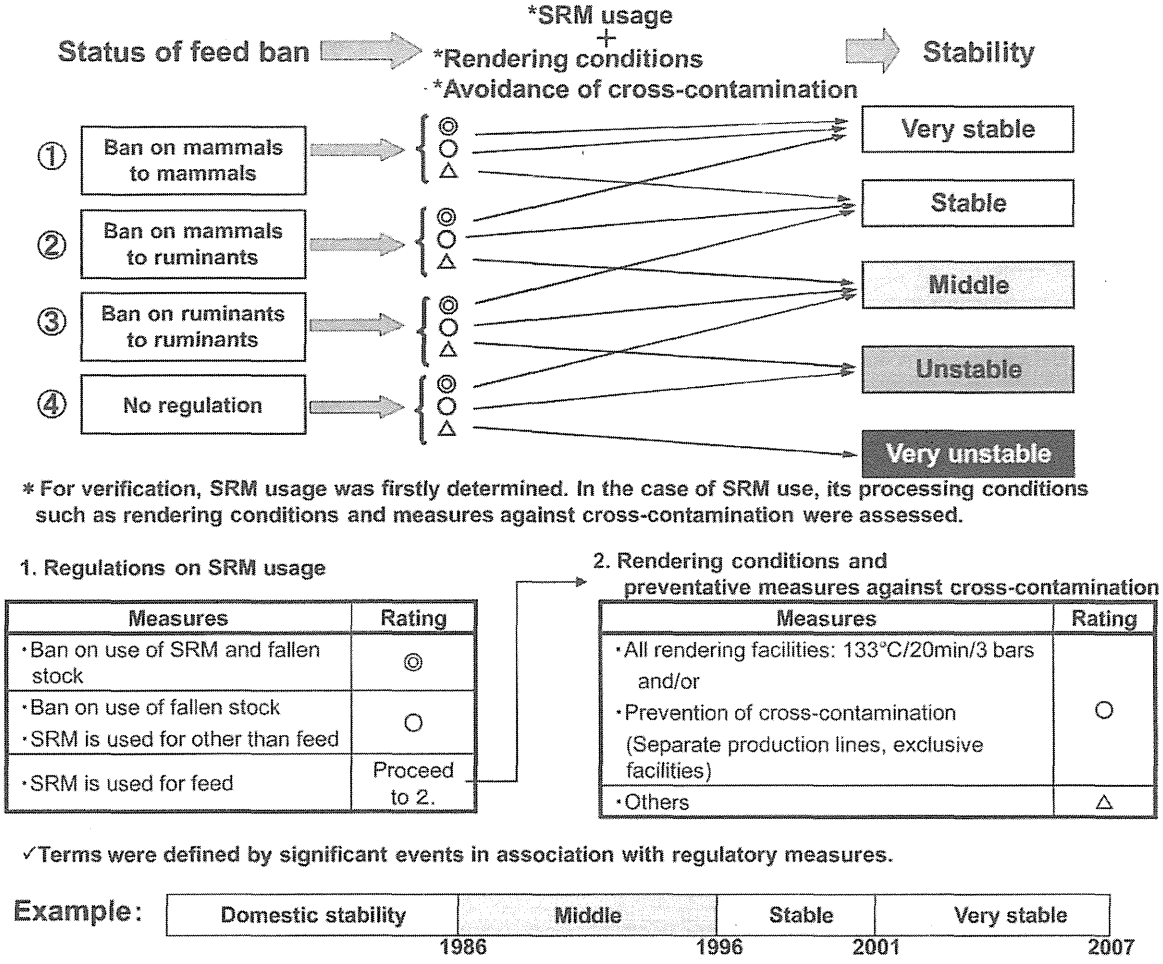


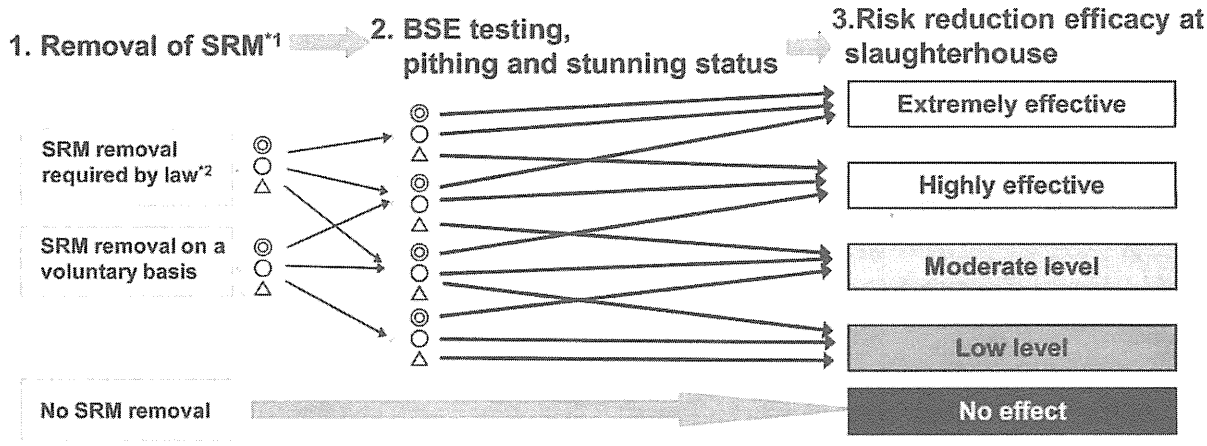
Fig. 2. Assessment of domestic stability. Each country was evaluated by the range of subjected animals and rigidity of the feed ban, followed by SRM-related conditions, to be assigned to one of the 5 ranks indicating degree of domestic stability. The rigidity of feed ban was rated based on the strengths of SRM-handling measures (the two lower boxes). At the bottom, an example of domestic stability is shown per terms defined by significant regulatory changes.

Pithing of animals at the slaughterhouse is linked to an increased risk of BSE contamination via brain and spinal tissue spillage from the stunning hole onto the processed meat and slaughtering facilities. It also increases the possibility of high-risk tissue (brain and spinal cord) leakage into the blood stream. Likewise, a stunning method with intracranial air/gas pressuring may also bring about a similar manner of contamination [1]. Therefore, the current assessment took into consideration slaughtering processes such as implementation of pithing or air/gas injection stunning in each assessed country.

Others (mechanically recovered meat, MRM, etc.): MRM including meat from advanced meat recovery (AMR) is meat obtained from bones by secondary recovery using mechanical techniques after primary removal of the major meat blocks. This method carries a certain risk of SRM contamination. Thus, the same assessment method as for primary beef meat blocks cannot be adopted for MRM in evaluation of the risk-reducing efficacy of BSE measures. Accordingly, the Commission requested the assessed countries known for MRM production to submit additional information regarding the SRM definition, measures and MRM exportation to Japan, and then these data were assessed separately from general beef and beef offal exports.

The total number of livestock and their traceability were also requested as supplementary data because they are related to the sensitivity and precision of antemortem inspection at the slaughterhouse or estimation of animal age in months.

Overall assessment of risk-reducing measures at meat processing lines: The process of assessment for efficacy of risk reduction during meat processing is concisely demonstrated in Fig. 3, aided by the explanation as follows. The efficacy of risk reduction during meat processing was assessed firstly by the degree of SRM removal. In the case that SRM removal was conducted in accordance with regulations, the risk reduction level was regarded as the highest, followed by voluntary removal; no regulation was the worst grade. Secondly, the actual measures used for SRM removal were



*1 Judgment was based on the conditions of SRM removal and procedures at slaughterhouses (If available, data of actual compliance was also taken into consideration.)
*2 Removal by domestic regulation, or by additional conditions required for exportation to Japan

1. SRM removal

Measures	Rating
① Confirmation by meat inspector ② Carcasses washed by high pressure water ③ Washing of the saw between carcasses ④ SSOP and HACCP regulations	◎
3 or 4 of the above measures implemented	
2 of the above measures implemented	○
Others	△

2. BSE testing, stunning and pithing status

Measures	Rating
・Health inspection to eliminate downers and BSE testing at slaughterhouses and ・Ban on air pressure stunning and pithing	◎
Both measures implemented	
One measure implemented	○
Others	△

Fig. 3. Flow chart of the assessment for efficacy of risk reduction at slaughterhouses. Each county was firstly reviewed for the status of SRM removal, followed by the rating of other risk-avoiding measures, to determine its risk reduction efficacy.

verified based on the actual conditions for such things as meat inspector's process confirmation, washing of carcasses with high pressure water to assure the complete removal of central nervous tissues, washing of the saws between individual carcasses and regulation by a Sanitation Standard Operating Procedure (SSOP) and Hazard Analysis and Critical Control Points (HACCP). If 3 or 4 of the above measures were conducted, the risk reduction efficacy was given a one-rank promotion when proceeding to the next assessment point (◎). The second verification point for risk reduction was the combination of BSE testing and absence of pithing/air stunning. If health inspections to eliminate downers and BSE testing were conducted in the slaughterhouse together with bans for both air pressure stunning and pithing, the efficacy of risk reduction was promoted one rank (◎). If one of the above measures was conducted, the efficacy of risk reduction stayed at the same level (○);otherwise, the country was demoted one rank. Based on the above-mentioned principles, each assessed country was categorized according to 5 grades for the efficacy of its risk-reducing measures, namely no effect, low level, moderate level, highly effective and extremely effective (Fig. 3).

Since the current assessment subjected only the meat products imported by Japan, the scope of evaluation was focused on the criteria for beef product preparation and BSE-preventive measures intended for each country's exports to Japan. Presently, all the beef and beef offal importers in Japan are requested to voluntarily refrain from importing SRM from any foreign countries. Some exporting countries even have their own specific regulations on exclusion of SRM from beef and beef offal exports to Japan under the Animal Health Requirement requested from Japan. Therefore, these risk control measures were also taken into the assessment along with the information obtained from each country upon the Commission's request through a questionnaire.

RISK OF MECHANICALLY RECOVERED MEAT (MRM)

Among the countries that have submitted replies to Japan's inquiry, two countries reported domestic production of MRM, with one country having exported 81.6 kg of MRM (head parts not included in raw materials) to Japan in 2008, while the other had no such record.

The key structure of the current risk assessment was the evaluation of imported beef and beef offal to Japan, which consists of a combination of multiple aspects such as risk of live cattle and risk-reducing measures at slaughterhouse

and meat processing lines in fabrication plants. Accordingly, any commodities that do not fall into these categories, such as MRM, should be considered separately. There are so far no grounds to suggest that MRM contamination is not connected with SRM through meat processing lines. Thus, at least MRM from those countries that have potentially had exposure to and/or propagation of BSE in the immediate past according to the data collected should be regarded as carrying certain risks. However, MRM from countries that are regarded as having a negligible possibility of BSE exposure/propagation may be considered as carrying negligible risks, provided the precondition of the current assessment, namely classical BSE originating from the U.K., is appropriately met.

Recently, there have been a few cases of irregular forms of BSE (atypical BSE) reported apart from classical BSE in Europe, Japan and the U.S.A. These reports of atypical BSE indicated variation in molecular sizes of abnormal prion proteins (PrPSc) among cases, and eventually two major sizes of proteins were designated as the H and L types.

Most of the atypical BSE cases were found in aged cattle over 8 years old, but a remarkable exception exists in Japan, where a steer only 23 months old was reported to have been infected with atypical BSE (the 8th BSE case in Japan). When this exception was excluded, the detection ages of atypical BSE cases ranged from 6.3 to 18 years old. The average detection ages for the H and L types were 11.8 and 11.6 years old, respectively [3].

To the best of the authors' knowledge, there have been about 40 cases of atypical BSE reported worldwide, yet the OIE does not require distinction between classical and atypical BSE cases in member countries for their reports, while the EFSA only recently referred to case reporting by classical/atypical recognition in its 2009 scientific opinion. These situations seem to further obscure the clear number of atypical BSE cases occurring in the world.

The origin of atypical BSE has not yet been determined. According to EFSA's scientific opinion published in 2008, all the cases of atypical BSE were reported with birth dates before the real feed ban in January 2001 in Europe. Therefore, the possibility of these atypical cases being attributed to contaminated feeds, just as in classical BSE, cannot be completely denied. On the other hand, data of atypical BSE cases (both the H and L types) in France did not show any reasonable correlation between birth year and frequency of occurrence, as was indicated in classical BSE cases, thus raising the possible interpretation of atypical BSE being sporadic isolated cases of prion disease [3].

Based on the data accumulated in France, the frequencies of atypical BSE cases per 1 million tested adult cattle were estimated to be 0.41 and 0.35 cases for the H and L types, respectively (1.9 and 1.7 cases for the H and L-types, respectively, when limiting the sampling to tested cattle over 8 years old). In Japan, a total of 10 million cattle including fallen stock and slaughtered cattle were tested for BSE, and the results showed no positive cases of the H type and 2 positive cases (case 8, a 23-month-old steer; case 24, a 169-month-old Japanese black cow) of the L type of atypical BSE. These data indicate that Japan has prevalence frequencies of 0 and 0.2 cases of the H and L types of atypical BSE per 1 million cattle including tested fallen stock and slaughtered cattle (zero and approximately 1.5 cases of the H and L types respectively, when limiting the sampling to tested slaughtered cattle over 8 years old).

Atypical BSE of both the H and L types was confirmed to be transmissible by intracerebral inoculation in transgenic mice expressing alleles of bovine or ovine PrP genes and of inbred mice. However, for transgenic mice expressing human prion protein, the L type but not the H type could be transmitted according to the previously published reports (recently, it was reported that H type also transmissible to the humanized transgenic mice). There have also been reports of glycosylation pattern transformation from L-type BASE3 PrP^{Sc}-like type to more of the classical BSE PrP^{Sc} type. This phenomenon was observed during passage using inbred and transgenic TgVR2 mice. As for the atypical cases of BSE confirmed in Japan, the 24th case of BSE was determined to have had the atypical L type at the detection age of 169 months old, and its sample was successfully transmitted to transgenic mice expressing bovine prion protein. However, transmission of a sample from the other case of atypical L-type BSE confirmed in Japan (the 8th case; detected at the age of 23-month-old) was reported to be unsuccessful in transgenic mice expressing bovine prion protein. The reason for this inconsistency is not clear at this time, although the possible presence of a limitation in the amount of prion protein accumulated in the subject's brain sample or that the inoculated volume was too low to reach the detection limit cannot be excluded.

A recent report has shown that the atypical L type of BSE has a higher degree of potential for pathogenicity than that its classical counterpart because incubation periods are shorter in atypical BSE transmitted to transgenic mice expressing human prion protein, suggesting that atypical BSE possibly has a higher degree of pathogenicity when compared to its classical counterpart [7].

In contrast to classical BSE, the systemic distribution of abnormal prion protein in atypical BSE cases is barely known. Therefore, it is unclear whether the brainstem is truly the optimal part for sampling and testing in H/L type detection. Likewise, information regarding the infectivity distribution of atypical BSE is scarce in bovine peripheral tissues and body fluid. All together, the lack of essential data hinders, to a certain extent, evaluation of the relative risk-reducing effects of various SRM removal measures for cattle.

Based on the currently available data concerning the potential risks for humans of atypical BSE and prevalence of atypical BSE, it may be too extreme to deny the risk of MRM, especially in MRM derived from aged cattle. However, the degree of influence of the presence of atypical BSE on our concept of the MRM risk will be limited to a low level under the circumstances with presently available knowledge and our discussion. In the meantime, one must also be reminded of the fact that only a limited amount of data is currently available concerning atypical BSE. A proper amount of dis-

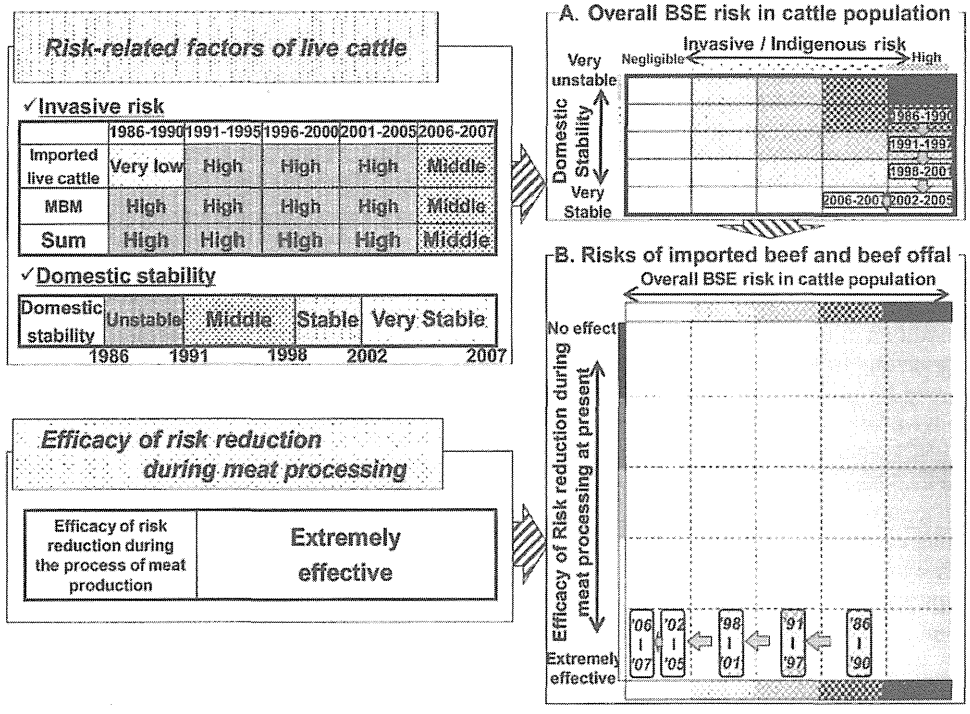


Fig. 4. Overall assessment results in a schematic view. A: The shift of overall BSE risks among age cohorts. The total invasive risks (the sum of imported live cattle and MBM risks; vertical axis) were plotted against domestic stability ranks (horizontal axis) in 2-D rank plot A. Note that the horizontal axis partially refers to the indigenous risk (domestic BSE propagation), which is also a reflection of invasive risk and domestic stability. Therefore, the results, shown in rank plot A, indicate the shift of BSE infection risks among the birth cohorts. B: The age-specific BSE risks in rank plot A and the efficacy of risk reduction during meat processing were combined. The outcome, 2-D rank plot B, was used to supplement the assessment process to get the final conclusion for each assessed country.

cretion should be used when interpreting these data to avoid unnecessary confusion. Further research and accumulation of data will bring additional insight into the mechanism, pathogenicity and transmission potential of atypical BSE, for which further assessment may become necessary in the future.

RISK ASSESSMENT OF BEEF AND BEEF OFFAL IMPORTED INTO JAPAN

To gain the final result of this assessment, the periodic BSE risk status of a country (the sum of invasive BSE risk and domestic stability) and efficacy of present BSE risk-reducing measures at meat processing lines were combined and used as an indicator of comprehensive likelihood of BSE prion contamination in beef and beef offal imported into Japan. Surveillance data were used to verify reliability of the assessment. Finally, a summary of each country was expressed in schematic figures (an example is shown in Fig. 4).

In Fig. 4, a model country's invasive risk was ranked as high (from 1986–2005) but was reduced to the middle level from 2006 onwards. The efficacy of feed ban (domestic stability) was unstable during 1986–1989 but improved to the middle level (1990–1996), to the stable level (1997–2000) and then to the very stable level (2001-until now). Current risk reduction efficacy at meat processing lines, determined by factors such as the definition of SRM, compulsory removal of SRM by law, and HACCP/SSOP procedures were good and verified and were therefore rated as ◎. BSE testing at slaughterhouse (>30 months), proper slaughtering procedures such as avoidance of air stunning and pithing were verified as ◎. All together, the overall risk reduction was extremely effective.

The final assessment for this model country was as follows: the domestic BSE exposure/propagation risk was low, and risk reduction at meat processing lines was extremely effective; therefore, the risk of BSE contamination of beef and beef offal imported from this assessed country was considered to be negligible.

ACKNOWLEDGMENTS. This work was conducted by the PEC of the FSC in Japan. The members of the PEC appreciate the FSC staff for their laborious efforts in data collection and processing, Dr. Ruriko Nakao for her assistance in manuscript preparation and the FSC counterparts of the assessed counties for their data contribution. Without their sincere help, we could not complete this work.

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THE JOURNAL OF IMMUNOLOGY

Cutting Edge: *Brucella abortus* Exploits a Cellular Prion Protein on Intestinal M Cells as an Invasive Receptor

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Cutting Edge: *Brucella abortus* Exploits a Cellular Prion Protein on Intestinal M Cells as an Invasive Receptor

Gaku Nakato,*,† Koji Hase,*,† Michio Suzuki,‡ Masanobu Kimura,‡ Manabu Ato,§ Misaho Hanazato,*,† Minoru Tobiume,¶ Motohiro Horiuchi,‖ Ryuichiro Atarashi,# Noriyuki Nishida,# Masahisa Watarai,** Koichi Imaoka,‡ and Hiroshi Ohno*,†

Brucella abortus is a Gram-negative bacterium causing brucellosis. Although _B. abortus_ is known to infect via the oral route, the entry site in the gastrointestinal tract has been unclear. We found that _B. abortus_ was selectively internalized by microfold cells (M cells), a subset of epithelial cells specialized for mucosal Ag uptake. During this process, colocalization of cellular prion protein (PrP^C) and _B. abortus_ was evident on the apical surface as well as in subapical vacuolar structures in M cells. Internalization of _B. abortus_ by M cells of PrP^C-deficient ($Prnp^{-/-}$) mice was greatly reduced compared with that in wild-type mice. Furthermore, an oral infection study revealed that translocation of _B. abortus_ into the Peyer's patch was significantly lower in $Prnp^{-/-}$ than in wild-type mice. These observations suggest that orally infected _B. abortus_ invades the host through M cells by using PrP^C on the apical surface of M cells as an uptake receptor. _The Journal of Immunology_, 2012, 189: 1540–1544.

The mucosal surface of the gastrointestinal tract is continuously exposed to vast numbers of commensal microorganisms and sporadically to pathogens. In this context, GALT such as Peyer's patches (PPs) serve as sentinels for the recognition and initiation of the immune responses against those microbes (1). One of the unique features of GALT is the lack of afferent lymphatic ducts, which necessitates the sampling of luminal Ags across the mucosal epithelium. The luminal side of the GALT lymphoid follicles is covered by the dome-shaped follicle-associated epithelium (FAE), within which are microfold cells (M cells). M cells are a unique subset of epithelial cells that actively transport luminal macromolecules through transepithelial membrane traffic, a process referred to as transcytosis (2, 3). Luminal contents transported via M cells are in turn captured by dendritic cells (DCs) residing beneath M cells to initiate mucosal immune responses, which ultimately leads to the production of Ag-specific IgA by B cells (1). Ag delivery through M cells is thus important for host defense. In contrast, the M cell-dependent Ag uptake process can be exploited by diverse pathogenic microbes, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) and *Yersinia enterocolitica* as a portal for host invasion (4). Proteins and/or oligosaccharides on the M-cell apical surface, including GPI-anchored proteins (5), are postulated to serve as receptors for these pathogens (6). In this regard, we have recently shown that the GPI-anchored protein gp2 (GP2) is specifically expressed on the apical plasma membrane of M cells and serves as an endocytic receptor for *S.* Typhimurium and *Escherichia coli* (7). We have also discovered that another GPI-anchored protein, cellular prion protein (PrP^C), is predominantly expressed on the M-cell apical surface among the intestinal epithelial cells (8), suggesting its role as a similar endocytic receptor.

Brucella abortus is a Gram-negative bacterium that causes brucellosis, a major zoonotic infection. Brucellosis manifests as undulant fever, arthritis, endocarditis, and meningitis in humans, as well as abortion and infertility in domestic and wild animals. *B. abortus* is a facultative intracellular pathogen that replicates within both phagocytic and nonphagocytic host cells (9). The organism is taken up by macrophages through a process involving initial movement on the cell surface and generalized membrane ruffling, leading to swimming internalization (10). The internalized *B. abortus* are enclosed by phagosomes with accumulated lipid rafts to form replicative vacuoles that do not fuse with lysosomes (11). The Type IV secretion system (T4SS) encoded by the *VirB* genes appears to

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Abbreviations used in this article: DC, dendritic cell; FAE, follicle-associated epithelium; GP2, gp2; h, human; Hsp60, heat shock protein 60; m, mouse; M cell, microfold cell; PP, Peyer's patch; $Prnp^{-/-}$, PrP^C-deficient; PrP^C, cellular prion protein; T4SS, Type IV secretion system; WT, wild-type.

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be essential for replicative vacuole formation, because phagosomes containing a *virB* mutant strain of *B. abortus* fuse with lysosomes to form conventional phagolysosomes (12). The inhibition of phagolysosome formation by T4SS has thus been implicated as a mechanism for the intracellular survival of *B. abortus*. Of interest, *B. abortus* expresses heat shock protein 60 (Hsp60) on its cell surface, probably via T4SS-mediated secretion. The surface-expressed Hsp60 binds to the PrP^C on macrophages (13). This interaction facilitates macropinosome formation and subsequent intracellular replication of *B. abortus* within macrophages. Although the above-mentioned in vitro studies have revealed the intracellular survival mechanisms of *B. abortus,* the in vivo infectious route of this bacterium is still unclear. *B. abortus* is classified as a food-borne pathogen; however, it remains to be elucidated how this bacterium can translocate across the mucosal epithelial barrier.

In this article, we report that *B. abortus* is efficiently internalized only into M cells among intestinal epithelial cells, suggesting a role for M cells as an entry portal for this bacterium after oral infection. We also observed colocalization of PrP^C and *B. abortus* on the apical surface of M cells. Importantly, the translocation of *B. abortus* into PPs after oral administration was significantly reduced in PrP^C-deficient mice. These observations indicate that PrP^C on M cells serves as a major uptake receptor for *B. abortus* during oral infection.

Materials and Methods

Animals

BALB/c and C57BL/6 mice were purchased from CLEA Japan. $Prnp^{-/-}$ mice (14) were backcrossed onto a C57BL/6 background. Other PrP^C-deficient ($Prnp^{-/-}$) mice (RBRC00437) were provided by RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan (15). These mice were maintained under specific pathogen-free conditions. Animal experiments were approved by the Animal Research Committees of all institutions.

Recombinant mouse prion protein preparation

To obtain constructs for fusion proteins of mouse (m) PrP^C ($mPrP^C$) with the Fc segment of human (h) IgG_1 ($hIgG_1$) (mPrP–Fc), cDNA prepared from FAE was used as a template for PCR amplification. Primers used were as follows: 5′-CGGGATCCACCATGGCGAACCTTGGCTACT-3′ (forward) and 5′-CGCTCGAGGGATCTTCTCCCGTCGTAATAG-3′ (reverse) cDNA fragments were inserted into the BamHI/XhoI cloning sites of a pcDNA3 expression vector (Invitrogen) containing a fragment encoding the Fc segment of $hIgG_1$, to obtain mPrP–Fc. Recombinant protein was prepared as described (7).

In vitro Brucella *spp. binding assay*

A total of 0.25 μg mPrP–Fc or control hIgG–Fc proteins were immobilized in 96-well flat-bottom plates (Nunc) by incubation overnight at 4°C. After washing, the wells were incubated with 1% BSA in PBS for 2 h for blocking, and then incubated for 2 h with 1×10^6 CFUs *B. abortus* 544 at 25°C. For Ab blocking, *B. abortus* were incubated with 0.5 μg/ml anti-Hsp60 Ab (Enzo Life Science) or isotype-matched control Ab (Jackson Immunolaboratory) for 15 min before binding assay. After washing five times with sterile PBS, genomic DNA was extracted from bound bacteria with a DNeasy Blood & Tissue Kit (Qiagen). Quantitative PCR was performed to quantify copy numbers of *bcsp31* (16), using the SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (TAKARA).

Ligated intestinal loop assay

Mice were anesthetized with Avertin (0.4 mg/g) and placed on a warming pad during the procedure. Next, 50 μg/ml mPrP mAb (SAF-32; Cayman Chemical) or isotype-matched control IgG (BD Biosciences) was injected into the ligated intestinal loop containing PPs. After incubation for 30–60 min, PPs were excised and fixed with Cytofix/Cytoperm (BD Biosciences) for 1 h at 4°C. Intracellular localization of primary Abs was probed with 10 μg/ml Alexa Fluor 488-conjugated anti-mouse IgG Ab (Molecular Probes). The specimens were further treated with 20 μg/ml Rhodamine *Ulex europaeus* agglutinin-1 (UEA-1) (Vector Laboratories).

To assess the blocking effect of anti-Hsp60 Ab on *B. abortus* uptake by M cells, 1 million GFP–*B. abortus* (17), *B. abortus* 544, and *B. abortus* 544 pretreated with anti-Hsp60 Ab were injected into a ligated intestinal loop of C57BL/6 or $Prnp^{-/-}$ mice (14). After incubation, whole-mount specimens of PPs were immunostained with *B. abortus*-specific rabbit antisera (1:100 dilution) (17), together with anti-PrP mAb (44B1; Ref. 18) or GP2 mAb, followed by Alexa Fluor 594-conjugated anti-rat IgG. The specimens were analyzed with a DeltaVision Restoration deconvolution microscope (Applied Precision).

Evaluation of oral infection

C57BL/6 or $Prnp^{-/-}$ mice (four mice per group), 8–10 wk old were anesthetized with isoflurane or 50 mg/kg sodium pentobarbital. Then, mice were inoculated intragastrically by gavage with 0.2 ml 0.1 M sodium bicarbonate containing 1 million *B. abortus* 544. After 4 h, PPs were dissected and incubated at 25°C in sterile PBS containing 20 μg/ml gentamicin for 30 min. The tissues were weighed and homogenized in sterile PBS. The homogenates were plated on Thayer–Martin Selective Agar (BD) to determine CFUs.

Statistics

Statistical analysis was performed with the Mann–Whitney *U* test. Differences were considered significant at $p < 0.01$.

Results and Discussion

PrP^C on M cells serves as an endocytic receptor

Given that PrP^C is a GPI-anchored protein highly expressed on the M-cell apical surface (8) and that diverse infectious agents often use GPI-anchored proteins to gain entry into host cells (5), we hypothesized that it serves as an endocytic receptor for Ag sampling by M cells. This possibility was first examined by means of an in vivo Ab-uptake assay. We injected an anti-PrP mAb into a ligated intestinal loop containing PPs, to explore whether the mAb bound to PrP^C on the M-cell surface is internalized into M cells. The subcellular localization of the mAb was analyzed by deconvolution microscopy to obtain high-resolution images. Serial X–Y images demonstrated that the PrP mAb was efficiently internalized into vesicular structures in the cytoplasm of M cells (Fig. 1A, 1B). Internalization of the PrP mAb was observed only in M cells, and not in the surrounding FAE cells. It is unlikely that the Ab uptake was mediated by a nonspecific pinocytic pathway, because no internalization of an isotype-matched

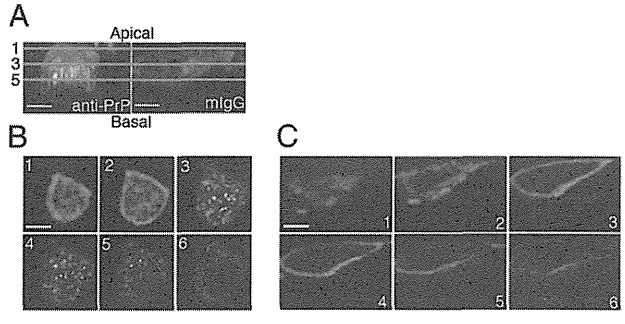

FIGURE 1. Anti-PrP mAb is taken up by murine PP M cells. A PrP mAb or isotype-matched control IgG (mIgG) was injected into the ligated intestinal loop. After incubation, PPs excised from the loop were subjected to whole-mount staining with fluorescent-conjugated secondary Ab to visualize the PrP mAb (green). The specimen was counterstained with UEA-1 (red) and then analyzed using DeltaVision deconvolution microscopy. (**A**) The X-Z image of the M cells. (**B** and **C**) Serial X–Y images from the apical (**1**) to the basal plasma membranes (**6**) of M cells shown in (A). The positions of X–Y images (1), (**3**), and (**5**) are indicated in (A). Scale bars, 5 μm.

control IgG took place at all (Fig. 1A, 1C). These observations suggest that PrP^C on M cells can serve as an endocytic receptor for the luminal constituents to which it can bind.

B. abortus *are selectively taken up by M cells through interaction with* PrP^C

Because PrP^C plays an important role in the uptake of *B. abortus* by macrophages (13), we examined whether this is also the case in M cells. Our in vitro binding assay using a rmPrP^C–hgG–Fcγ fusion protein (mPrP^C–Fc) confirmed the interaction between PrP^C and *B. abortus* (Fig. 2A). To further examine the interaction between PrP^C and Hsp60 on *B. abortus*, the bacteria were pretreated with anti-Hsp60 Ab before the binding assay. The binding efficiency of *B. abortus* to mPrP^C–Fc was profoundly impaired in anti-Hsp60–treated *B. abortus* compared with that in bacteria not treated or pretreated with isotype-matched control Ab. (Fig. 2B).

We then asked whether PrP^C expressed on M cells binds the bacterium. The ligated intestinal loop assay verified that *B. abortus* bound exclusively to M cells among epithelial cells in FAE and villous regions (Fig. 2C and data not shown). In addition, the X-Z images indicated that *B. abortus* were internalized into the cytoplasm of M cells, where colocalization of the *B. abortus* and PrP^C was evident (Fig. 2D). These results support the idea that *B. abortus* can be taken up by M cells through its interaction with PrP^C. To further confirm this possibility, we tested whether ablation of PrP^C affects the efficiency of *B. abortus* uptake by M cells. In the ligated intestinal loop assay, we found that the number of surface-bound *B. abortus* in PrP^C-deficient ($Prnp^{-/-}$) mice was less than half that in wild-type (WT) mice (Fig. 3A, 3B). Moreover, the internalization of *B. abortus* into M cells was markedly reduced in $Prnp^{-/-}$ compared with WT mice (Fig. 3B). We also examined the effect of anti-Hsp60 Ab on the interaction between PrP^C on M cell and *B. abortus*. Binding and internalization of *B. abortus* to M cells were decreased in anti-Hsp60–treated *B. abortus* (Fig. 3C). Taken together, these observations suggest an important role for PrP^C, via interaction with Hsp60 on the bacterial surface, in the entry of *B. abortus* into M cells.

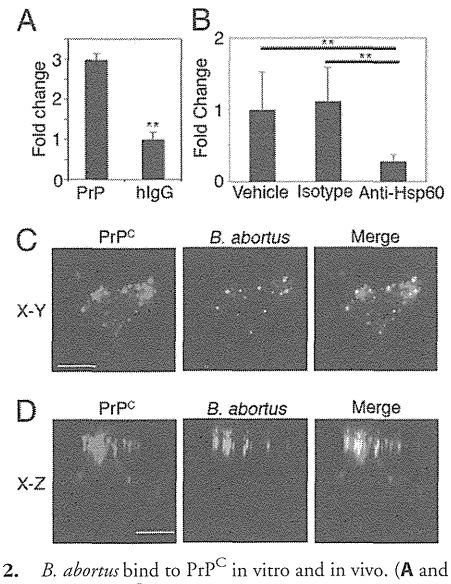

FIGURE 2. *B. abortus* bind to PrP^C in vitro and in vivo. (**A** and **B**) In vitro binding assay with rmPrP^C–Fc or control hIgG–Fc protein. *B. abortus* were pretreated with anti-Hsp60 Ab or isotype-matched control Ab before the interaction with the mPrP–Fc. Data are means and SE ($n = 3$). $^{**}p < 0.01$. (**C** and **D**) GFP–*B. abortus* were injected into the ligated intestinal loop of anesthetized C57BL/6 mice. After incubation, PPs excised from the loop were subjected to whole-mount staining with *B. abortus*-specific antisera (green) and PrP mAb (red), and then analyzed using a DeltaVision deconvolution microscope. The X–Y (C) and X–Z (D) images of M cells are shown. Scale bars, 5 μm.

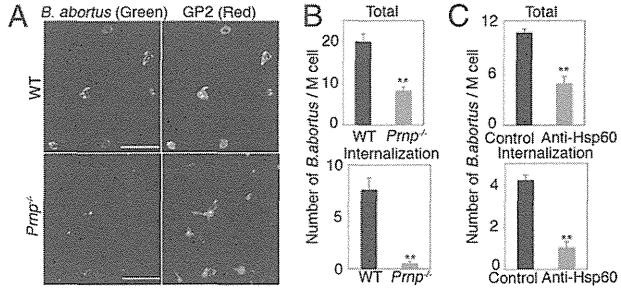

FIGURE 3. Decreased uptake of *B. abortus* by PP M cells in $Prnp^{-/-}$ mice. (**A**) GFP–*B. abortus* were injected into the ligated intestinal loop of anesthetized WT or $Prnp^{-/-}$ mice. After incubation, PPs excised from the loop were subjected to whole-mount staining with *B. abortus*-specific antisera (green) and GP2 mAb (red), and then analyzed using a DeltaVision deconvolution microscope. The X–Y images of FAE in WT mice (*upper panels*) and $Prnp^{-/-}$ (*lower panels*) are shown. Scale bars, 20 μm. (**B**) Quantitative analysis of the number of *B. abortus* associated with the apical surface of M cells and internalized into M cells between WT and $Prnp^{-/-}$. (**C**) Quantitative analysis of the number of *B. abortus* associated with the apical surface of M cells and internalized into M cells in the presence or absence of anti-Hsp60 Ab pretreatment. Data are expressed as the mean ± SD of 15 different samples for each group. $^{**}p < 0.01$.

B. abortus *enters the host via M cells*

To gain further evidence for PrP^C-dependent uptake of *B. abortus*, we performed oral infection with *B. abortus* in $Prnp^{-/-}$ and WT mice. After oral administration, a substantial number of viable *B. abortus* organisms were detectable in PPs of WT mice; by contrast, the bacteria were nearly undetectable in $Prnp^{-/-}$ mice (Fig. 4). Taken together with the above observations made with the ligated loop assay, this result underscores the biological significance of PrP^C in the uptake of *B. abortus*, as well as supports the idea that *B. abortus* enters the host through M cells, using PrP^C.

Prion protein is the causative agent of the transmissible spongiform encephalopathies. According to the "prion hypothesis," the infectious isoform of prion protein, termed

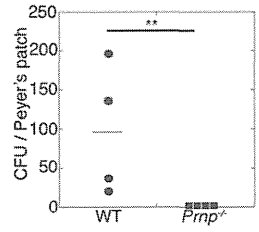

FIGURE 4. *B. abortus* infection through the gastrointestinal tract in WT and $Prnp^{-/-}$ mice. C57BL/6 or $Prnp^{-/-}$ mice were fed 1×10^6 CFU of *B. abortus*. After 4 h, bacterial translocation to Peyer's patches was examined. The horizontal solid line shows the average. Data are expressed as the mean ± SD of four different samples for each group. Representative data of three independent experiments are shown. $^{**}p < 0.01$.

PrP^{Sc}, replicates by interacting with cellular PrP^{C} and mediating its conformational change into the disease-causing PrP^{Sc} (19). Compared with its well-defined pathological significance, the physiological function of PrP^{C} remains unclear. PrP^{C} is highly expressed not only by cells in the CNS but also by follicular DCs, mature myeloid cells, and activated T cells. This distribution suggests involvement of PrP^{C} in immune surveillance (20).

Our present study defines a novel role for PrP^{C} as an M-cell receptor for the uptake of pathogenic bacteria. PrP^{C} on macrophages has been reported to recognize surface-exposed Hsp60 of *B. abortus* and to facilitate internalization of the bacteria (13); however, Fontes et al. (21) reported a contradictory result. Using $Prnp^{-/-}$ mice, they showed that *B. suis* infection is independent of PrP^{C} expression. By contrast, WT macrophages had a greater tendency to be infected with *B. abortus* than did $Prnp^{-/-}$ macrophages, although no significant difference between WT and $Prnp^{-/-}$ macrophages was found in intracellular multiplication of *B. abortus*. To shed more light on these observations, we performed oral infection with *Brucella* spp. in WT and $Prnp^{-/-}$ mice. After oral administration, a substantial number of *B. suis* organisms were detectable in both WT and $Prnp^{-/-}$ PPs (data not shown), whereas the translocation of *B. abortus* into PPs was significantly reduced in $Prnp^{-/-}$ mice (Fig. 4). These data are consistent with the observation by Fontes et al. (21). In addition, these authors discussed the spatial proximity of *Brucella* spp. and PrP^{C} during the early stage of infection. In accordance, our ligated loop assay showed that the internalized *B. abortus* were surrounded by PrP^{C} in the cytoplasmic vacuolar compartment of M cells (Fig. 2D). Taken together, these results suggest that *B. abortus* is efficiently taken up by M cells in a PrP^{C}-dependent manner. However, *B. suis* might invade the host independently of PrP^{C}.

Interaction of PrP^{C} and Hsp60 family proteins has been demonstrated by several approaches, including a yeast two-hybrid screening as well as a pull-down assay (22). Our in vitro binding assay confirmed the interaction between PrP^{C} and Hsp60 on *B. abortus* (Fig. 2B). We also showed that PrP^{C} on M cells interacts with Hsp60 on *B. abortus* (Fig. 3C). Nevertheless, we cannot formally exclude an alternative and mutually not exclusive possibility that PrP^{C} on M cells acts as a scaffold to coordinate several proteins in a complex, with the complex mediating *B. abortus* internalization (23). The Hsp60 proteins have been recognized as immunodominant Ags of many microbes (24). Hsp60 normally resides in the bacterial cytoplasm, but the protein can be secreted via T4SS and expressed on the outer membrane of the bacteria. In fact, the presence of Hsp60 on the bacterial surface is not restricted to *B. abortus*. A similar phenomenon has also been shown for other bacteria (25–28). The exposure of Hsp60 on the surface appears to increase bacterial adherence to host cells. PrP^{C} expressed on the apical plasma membrane of M cells in the GALT thus may contribute to immunosurveillance on the mucosal surface by promoting transcytosis of bacteria that express Hsp60 at their surface. This idea is concordant with the observation that *H. pylori* translocation from the intestinal mucosa into PPs, possibly via the M cells, is essential for the induction of humoral and cellular immunity against this pathogen (29).

Accumulating evidence supports the idea that many infectious agents and their toxins use GPI-anchored proteins to gain entry into host cells (5). For example, CD48 on macrophages and mast cells contributes to phagocytosis of *E. coli* via FimH recognition (30). On the basis of our observations that PrP^{C} and GP2 proteins on the apical plasma membrane of M cells can serve as receptors (this study and Ref. 7), M cells also seem to use GPI-anchored proteins for intestinal immunosurveillance. In conclusion, our findings indicate that the PrP^{C}-dependent route of bacterial uptake by M cells can be exploited for *B. abortus* invasion into the host. Once *B. abortus* penetrate M cells, the bacteria are capable of surviving inside DCs, which accumulate beneath the M cells, by forming replicative vacuoles with subsequent systemic spread to other organs. This model offers a new insight into the pathogenesis of *B. abortus* infection, a disease that leads to significant economic losses for cattle and other domestic animals and, in turn, transmission to humans. The disruption of the Hsp60–PrP^{C} interaction on the mucosal surface may provide a useful therapeutic target for protection against *B. abortus* infection.

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Disclosures

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