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 %			Frequency %				Frequency %			Frequency %				
Cattle (*n*)	ins	del	χ^2-value (degree of freedom)	ins/ins	ins/del	del/del	χ^2-value (degree of freedom)	ins	del	χ^2-value (degree of freedom)	ins/ins	ins/del	del/del	χ^2-value (degree of freedom)	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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References

Asante, E. A., J. M. Linehan, M. Desbruslais, S. Joiner, I. Gowland, A. L. Wood, J. Welch, A. F. Hill, S. E. Lloyd, J. D. F. Wadworth, and J. Collinge, 2002: BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J.* 21, 6358–6366.

Belt, P. B. G. M., I. H. Muileman, B. E. C. Schreuder, J. B. Ruijter, A. L. J. Gielkens, and M. A. Smits, 1995: Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *J. Gen. Virol.* 76, 509–517.

Bruce, M. E., R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, and C. J. Bostock, 1997: Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 389, 498–501.

Garcia, O., T. Hemme, L. T. Nho, and H. T. H. Tra, 2006: The Economics of Milk Production in Hanoi, Vietnam, with Particular Emphasis on Small-Scale Producers. PPLPI Working Paper No. 33. Published by Pro-Poor Livestock Policy Initiative, the Food and Agriculture Organization, Rome, Italy.

Hill, A. F., M. Desbruslais, S. Joiner, K. C. L. Sidle, I. Gowland, and J. Collinge, 1997: The same prion strain causes vCJD and BSE. *Nature* 389, 448–450.

Juling, K., H. Schwarzenbacher, J. L. Williams, and R. Fries, 2006: A major genetic component of BSE susceptibility. *BMC Biol.* 4, 33.

Kashkevich, K., A. Humeny, U. Ziegler, M. H. Groschup, P. Nicken, T. Leeb, C. Fischer, C.-M. Becker, and K. Schiebel, 2007: Functional relevance of DNA polymorphisms within the promoter region of the prion protein gene and their association to BSE infection. *FASEB J.* 21, 1547–1555.

Luthi, N. B., L. Fabozzi, P. Gautier, P.-Q. Trung, and D. Smith, 2006: Review, Analysis and Dissemination of Experiences in Dairy Production in Vietnam. Final Draft Report. Published by Pro-Poor Livestock Policy Initiative, the Food and Agriculture Organization, Rome, Italy.

Nakamitsu, S., T. Miyazawa, M. Horiuchi, S. Onoe, Y. Ohoba, H. Kitagawa, and N. Ishiguro, 2006: Sequence variation of bovine prion protein gene in Japanese cattle (Holstein and Japanese Black). *J. Vet. Med. Sci.* 68, 27–33.

Sander, P., H. Hamann, I. Pfeiffer, W. Wemheuer, B. Brenig, M. H. Groschup, U. Ziegler, O. Distl, and T. Leeb, 2004: Analysis of sequence variability of the bovine prion protein gene (*PRNP*) in German cattle breeds. *Neurogenetics* 5, 19–25.

Sander, P., H. Hamann, C. Drögemüller, K. Kashkevich, K. Schiebel, and T. Leeb, 2005: Bovine prion protein gene (*PRNP*) promoter polymorphisms modulate *PRNP* expression and may be responsible for differences in bovine spongiform encephalopathy susceptibility. *J. Biol. Chem.* 280, 37408–37414.

Schmick, H., and B. T. Huong, 2003: Vietnam Livestock and Products Swine and Dairy Sector Update 2002. GAIN Report VM3001. Published by USDA Foreign Agricultural Service, Hanoi, Vietnam.

Scott, M. R., R. Will, J. Ironside, H.-O. B. Nguyen, P. Tremblay, S. J. Dearmond, and S. B. Prusiner, 1999: Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc. Natl Acad. Sci. U.S.A.* 96, 15137–15142.

Seabury, C. M., J. E. Womack, J. Piedrahita, and J. N. Derr, 2004: Comparative PRNP genotyping of U.S. cattle sires for

potential association with BSE. *Mamm. Genome* 15, 828–833.

Smith, P. G., 2003: The epidemics of bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease: current status and future prospects. *Bull. World Health Organ.* 81, 123–130.

Wade, J., and N. T. Huong, 2005: Vietnam Livestock and Products Livestock and Products Update 2005. GAIN Report VM5056. Published by USDA Foreign Agricultural Service, Hanoi, Vietnam.

Wilesmith, J. W., J. B. M. Ryan, and M. J. Atkinson, 1991: Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet. Rec.* 128, 199–203.

FULL PAPER *Public Health*

Evaluation of Methods for Removing Central Nervous System Tissue Contamination from the Surface of Beef Carcasses after Splitting

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ABSTRACT. Since high levels of prions, the causative agent of bovine spongiform encephalopathy (BSE), accumulate in the brain and spinal cord, contamination of beef carcasses with central nervous system tissue (CNST) may occur at post-slaughter process. In this study, we investigated CNST contamination on the surface of beef carcasses using glial fibrillary acidic protein as a marker after splitting and evaluated the effects of washing procedures on contamination removal. High levels of CNST contamination was detected immediately after splitting, especially in the area close to the spinal column. This suggests that spinal cord fragments are attached to carcasses at the time of splitting even though the spinal cords have been removed by vacuum before splitting. Steam cleaning or manually washing with normal pressure water around the spinal column, performed prior to washing with high-pressure water, was found to be effective for reducing the level of CNST contamination. Furthermore, manually washing with high-pressure water could reduce CNST contamination to almost negligible levels. These results are useful for preparation of appropriate sanitation standard operating procedures to reduce the risk of CNST contamination of carcasses for prevention of exposure to BSE prion via the food chain.

KEY WORDS: BSE, carcass splitting, central nervous system tissues, glial fibrillary acidic protein, post-slaughter.

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Since the epidemiological linkage of bovine spongiform encephalopathy (BSE) to the occurrence of variant Creutzfeldt-Jakob disease in human was reported, BSE has been recognized as a foodborne disease [13]. Prion infectivity exists abundantly in the brain and spinal cord of BSE-affected cattle. BSE prion has also been detected in the trigeminal ganglion, dorsal root ganglia and, though at low levels, peripheral tissues such as the retina, bone marrow, tonsil and distal ileum [2, 11, 12]. The brain, spinal column including the spinal cord and dorsal root ganglia and distal ileum are removed as specified risk materials (SRMs) and thus kept out of the chain of consumption to reduce the risk of foodborne BSE infection. Recently, the existence of prion in tissues other than SRMs became evident; an abnormal isoform of prion protein, PrP^{Sc}, which is thought to be a major component of prion, was detected in the peripheral nerves [5] and adrenal glands of BSE-affected cattle [7].

There are reports of carcasses and meat products contaminated with CNST in spite of washing [6, 8, 14]. These reports determined the levels of CNST using glial fibrillary acidic protein (GFAP) as a marker and indicated that carcasses were contaminated with CNST under the following conditions: (1) brain tissue, destroyed by penetrating captive bolt stunning or pithing, is carried into muscle tissue via blood flow [1, 3]; (2) spinal cord fragments are attached to the surface of carcasses during splitting [4]; and (3) carcass washing after splitting is insufficient to remove spinal cord fragments from the carcass surface [8]. In addition, cross-contamination from SRM-contaminated carcasses is also a potential source of contamination [8].

In this collaborative study of 8 slaughterhouses in Japan, we investigated CNST contamination on the surface of the carcasses using GFAP as a marker for CNST. First, we investigated the contamination of carcasses with CNST immediately after splitting to understand the actual situation of contamination in Japan. Contamination was detected at all slaughterhouses examined, although the positive rate and degree of contamination varied among the slaughterhouses. Carcasses must be rid of CNST contamination in slaughterhouses before they are shipped as food products. Herein, we report the level of CNST contamination and effectiveness of washing procedures for removing CNST contamination on the beef carcass.

MATERIALS AND METHODS

Slaughtering process and analysis objectives: This study was conducted at eight domestic slaughterhouses (A-H) between August and November 2006. The general slaughtering process is outlined in Fig. 1A. Following stunning, exsanguination, and head removal, the carcasses were hung up by the hind legs. A tube was inserted into the spinal canal to suck out the spinal cord, and then the carcass was split. The carcasses were subsequently washed automatically or manually with high-pressure water. Prior to washing with high-pressure water, either steam cleaning or manual washing with normal-pressure water was carried out

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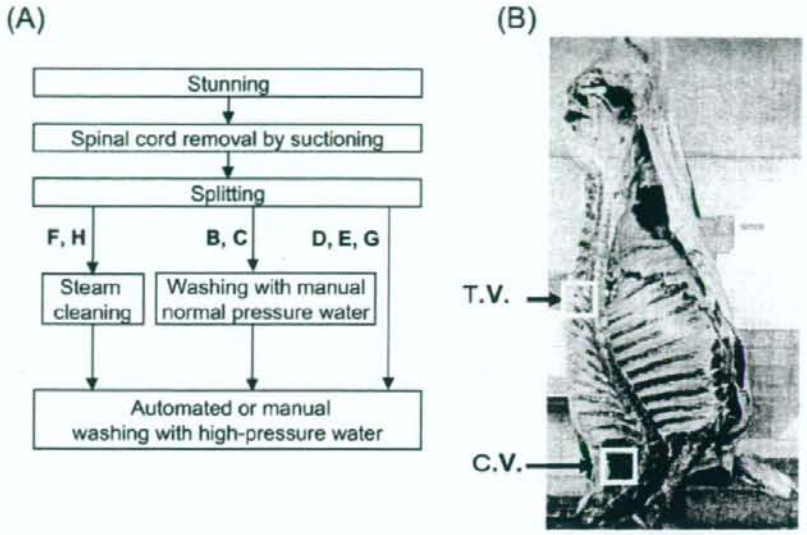

Fig. 1. Slaughtering process (A) and area for sampling (B). The effects of steam cleaning, washing with normal pressure water and manual washing with high-pressure water on the removal of CNST contamination from the surfaces of carcasses were evaluated at shaughterhouses F and H, B and C and D, E and G, respectively. C.V. and T.V. indicate the third cervical and last thoracic vertebra areas for sampling. One hundred cm^2 (10 cm x 10 cm) sections were wiped in the respective areas.

as preliminary washing to ensure the efficacy of high-pressure washing. At the time of planning of this research, most of slaughterhouses manually washed carcasses with high-pressure water without a preliminary washing step. Therefore, this study was designed to first investigate the actual situation of CNST contamination on the surface of carcasses immediately after splitting (at slaughterhouses A to H). Next, we attempted to evaluate the effects of steam cleaning (at slaughterhouses F and H) and manual washing with normal-pressure water (at slaughterhouses B and C) on CNST removal as a preliminary washing step and the effect of manual washing with high-pressure water on the CNST removal as the main washing step.

GFAP detection by enzyme immunoassay: One hundred square centimeters of sectioned muscle or fat on the split surface of wet carcasses were wiped off with dry sterile cotton applicators around the third cervical vertebra or last thoracic vertebra (Fig. 1B). The swabs of the applicators carrying the wiped-off materials were put into 2 m*l* sterile tubes for immediate laboratory testing.

Ridascreen Risk Material 10/5 (R-Biopharm GmbH, Germany), an ELISA-based test, was used to detect GFAP in the wiped-off samples. Following the manufacturer's instructions, the levels of CNST in the samples were determined from standard curves and were expressed as GFAP amounts per 100 cm^2. Samples with a GFAP concentration above 3 *ng*/100 cm^2, the detection limit of the kit used, were judged to be positive. Data analysis was performed assuming that GFAP concentrations below the detection limit were equal to 0 *ng*/100 cm^2. If the GFAP concentration exceeded the quantitation range, the sample was diluted for remeasurement and subsequent calculation of the actual GFAP concentration in consideration of the dilution ratio.

RESULTS

Residual CNST on the carcass surface immediately after splitting: Beef carcasses were investigated immediately after splitting in eight slaughterhouses to determine the levels of residual CNST on their surfaces (Table 1). Among these slaughterhouses, slaughterhouse D, which did not withdraw the spinal cord by suction, showed the highest GFAP positive rate (100%) around both the third cervical vertebra and last thoracic vertebra. The other slaughterhouses, at which the spinal cord was withdrawn by suction before splitting, showed GFAP positive rates ranging from 21.7 to 85.0%. In contrast, the residual levels of CNST exceeded 1 μg/100 cm^2 around the third cervical vertebra in slaughterhouse C and around the last thoracic vertebra in slaughterhouses B and H. This suggests that the level of contamination may not depend on removal of spinal cord, but instead scattering of saw residue during splitting may cause contamination in a limited area. Thus, removal of the scattered residue by washing is essential to reduce contami-

Table 1. Residual levels of CNST immediately after splitting

Slaughterhouse	Wiped section[a)]	n	Positive rate (%)	GFAP (ng/100 cm^2)			
				Mean	Standard deviation	Maximum	Minimum
A	C.V.	60	21.7	2.4	5.7	29.6	<3
	T.V.	60	73.3	22.3	31.4	178.3	<3
B	C.V.	20	85	89	150.3	651.8	<3
	T.V.	20	70	156.3	283.1	1,076.40	<3
C	C.V.	70	32.9	27.6	138.5	1,112.30	<3
	T.V.	70	42.9	19.4	80.2	578	<3
D	C.V.	111	100	49.6	49.1	303.5	3.3
	T.V.	111	100	149.6	132.6	490.9	10.2
E	C.V.	40	30	5.7	10.3	36.1	<3
	T.V.	40	22.5	12	28.2	121.8	<3
F	C.V.	55	43.6	8.7	26.5	190.6	<3
G	C.V.	40	45	9.8	27.9	168.7	<3
	T.V.	40	52.5	16.9	32	161.3	<3
H	C.V.	100	44	36.4	91	648.8	<3
	T.V.	100	24	32.2	152.2	1,328.40	<3

a) C.V. and T.V. indicate the third cervical and last thoracic vertebra areas, respectively.

Table 2. Effects of steam cleaning[a)]

Slaughterhouse	Wiped section[b)]	n	GFAP concentration (ng/100 cm^2)						Rate of decrease (%)
			Before steam cleaning			After steam cleaning			
			Mean	Maximum	Minimum	Mean	Maximum	Minimum	
F	C.V.	20	11.7	190.6	<3	3.4	51.2	<3	70.9
H	C.V.	100	36.4	648.8	3.9	13.6	348.2	3.4	62.6
	T.V.	100	32.2	1,328.4	3.4	18.4	493.0	3.5	42.9

a) Steam Vacuum System Model CV-1 (Jarvis, USA) was used.
b) C.V. and T.V. indicate the third cervical and last thoracic vertebra areas, respectively.

Table 3. Effects of manual washing with normal pressure water[a)]

Slaughterhouse	Wiped section	n	GFAP concentration (ng/100 cm^2)						Rate of decrease (%)
			Before washing			After washing			
			Mean	Maximum	Minimum	Mean	Maximum	Minimum	
B	C.V.	20	115.8	651.8	<3	21.7	73.9	<3	81.3
	T.V.	20	155.4	1,076.4	<3	17.2	116.8	<3	88.9
C	C.V.	10	115.1	1,112.3	<3	0.8	4.6	<3	99.3
	T.V.	10	63.1	578.0	<3	15.6	106.6	<3	75.3

a) The entire split surfaces of carcasses were washed evenly for 15 seconds.

nation of carcasses with CNST.

Effects of steam cleaning on CNST removal: The effects of steam cleaning on CNST removal were examined in slaughterhouses F and H. Steam cleaning was performed by spraying the surfaces of carcasses with approximately 80°C steam and simultaneously sucking substances attached to the surface of the carcasses through a suction nozzle moving across the carcass surface. Table 2 shows the mean levels of CNST residue before and after steam cleaning and the corresponding rate of decrease. In addition, the maximum and minimum levels of CNST residue are also indicated. In each slaughterhouse, the maximum levels of CNST residue decreased after washing. All cases showed a 42.9 to 70.9% decrease in the mean CNST levels after steam cleaning, demonstrating that this treatment is effective for removing CNST residue attached to carcasses.

Effects of manual washing with normal pressure water on CNST removal: The effects of manual washing with normal pressure water on CNST removal were examined in slaughterhouses B and C. The entire split surfaces of carcasses were washed evenly for 15 seconds. Table 3 shows the mean levels of residual CNST before and after washing and

the corresponding rate of decrease after washing. The maximum levels of CNST residue decreased after washing, and both slaughterhouses showed a decreases in the mean CNST levels of more than 75% after manual washing with normal pressure water, demonstrating that this treatment is also effective for CNST removal from carcasses.

Effects of manual washing with high-pressure water on CNST removal: In Japan, slaughterhouses generally conduct manual washing of carcasses with high-pressure water using a gun-type nozzle if automatic high-pressure washers are not available. At present, more than 60% of slaughterhouses have introduced manual washing with high-pressure water (unpublished observation). Therefore, the effects of manual washing with high-pressure water on CNST removal were examined in slaughterhouses D, E and G. Ebara Pressure washers (Ebara Corp., Japan), Plunger Pump units (Arimitsu Industry Co., Ltd., Japan) and Rocky washers (Arimitsu Industry Co., Ltd., Japan) were used in slaughterhouses D, E and G, respectively.

Figure 2 shows the changes in the average levels of CNST residue during manual washing with high-pressure water. In slaughterhouse D, the average residue level of CNST around the last thoracic vertebra was as high as 159.7 *ng*/100 cm^2 before washing, but decreased to 24.7 *ng*/100 cm^2 after washing for 15 seconds and then to 3.4 *ng*/100 cm^2 after washing for 60 sec. Similarly, the average CNST residue level around the third cervical vertebra was as high as 59.3 *ng*/100 cm^2 before washing, but decreased to 19.7 *ng*/100 cm^2 after washing for 15 sec and then to 4.8 *ng*/100 cm^2 after washing for 60 sec (Fig. 2A). In slaughterhouse E, the average CNST residue levels were relatively low before washing (12.1 *ng*/100 cm^2 around the last thoracic vertebra and 5.7 *ng*/100 cm^2 around the third cervical vertebra). The levels fell below the detection limit around the last thoracic vertebra after washing for 45 sec and around the third cervical vertebra after washing for 30 sec.

We also examined the efficacy of manual washing with high-pressure water in terms of washing order (Fig. 2B). In slaughterhouse G, the upper part of the split surfaces of carcasses in particular were washed for 40 sec and then the lower parts of the split surfaces of some of the carcasses were washed intensively for 30 or 45 sec. The average CNST levels around the third cervical vertebra before washing, 8.1 *ng*/100 cm^2, increased to 14.4 *ng*/100 cm^2 when only the upper part of the carcass was washed, but decreased below the detection limit when the lower part of the carcass was also washed for at least 45 sec. The average CNST residue level around the last thoracic vertebra, which was 7.1 *ng*/100 cm^2 before washing, decreased significantly even when only the upper carcass was washed and decreased below the detection limit when the lower section of the carcass was also washed.

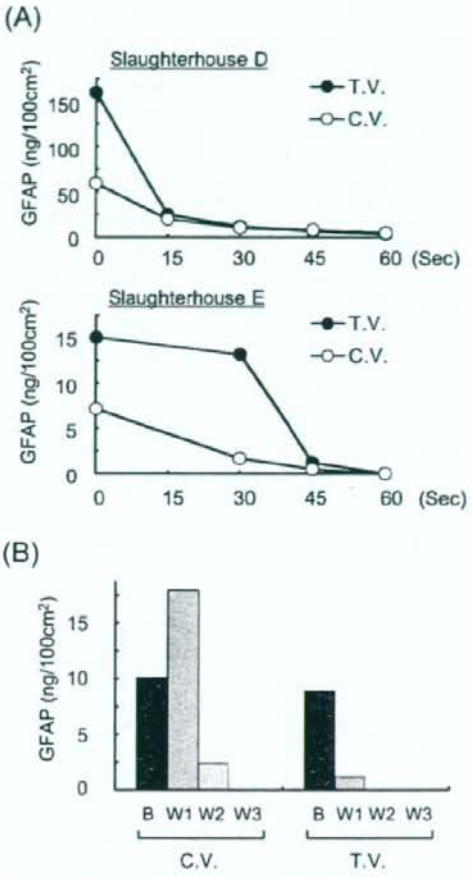

Fig. 2. Effect of manual washing with high-pressure water on removal of CNST contamination. (A) Evaluation of duration of washing. Zero indicates the GFAP levels before washing, and the GFAP levels after washing for 15 to 60 sec were plotted. The graphs show the average of 10 samples. C.V. and T.V. indicate the third cervical and the last thoracic vertebra areas for sampling. (B) Effect of the order of washing. Three washing protocols (W1 to W3) were tested for removal of CNST fragments from the carcasses. B, before washing; W1, washing only the upper parts of carcasses for 40 sec; W2, washing of the upper parts for 40 seconds and subsequent washing of the lower parts for 30 sec; W3, washing of the upper parts for 40 sec and subsequent washing of the lower parts for 40 sec. The graph shows the averages for 20 samples.

DISCUSSION

There are several reports available concerning CNST contamination on the surface of carcasses after high-pressure washing in slaughterhouses. Prendergast *et al.* [8] reported high contamination near the splitting line on the internal surfaces of carcasses, and Lim *et al.* [6] reported that the internal surfaces of carcasses were more contaminated than the external surfaces. According to their findings, they believe that contamination on the surface of

carcasses depends not only on the slaughtering method but also on the splitting or washing method. According to Prendergast *et al.* [9], the longer splitting takes, the larger the amount of sawing residue accumulation on the blade and thus the greater the CNST contamination of the carcass.

In the present study, we showed that the degree of CNST contamination was higher in a slaughterhouse where the spinal cord was not removed before splitting than in those where the spinal cord was removed before splitting. Thus, removal of the spinal cord before splitting is believed to be effective in reducing the occurrence of CNST contamination during splitting. Similarly, Helps *et al.* [4] reported that CNST contamination on the carcass surface is reduced by removal of the spinal column with an experimental oval saw that can remove it without exposing the spinal cord. These reports indicate that spinal cord suction plays a role in reducing the risk of CNST contamination of carcasses. However, it is difficult to completely remove the spinal cord by suction, and substantial amount of residual spinal cord and dura matter still remain in the spinal column after suction. In fact, we showed that the residual levels of CNST varied greatly from over 1 $\mu g/100\ cm^2$ to below the detection limit, even though the spinal cord was removed by vacuum before splitting. Such contamination on the surfaces of carcasses is possibly due to scattering of spinal cord fragments remaining in the spinal columns as sawing residue, as pointed out by Ramantanis [7]. Thus, adequate washing of carcasses after splitting is indispensable for reducing the risk of CNST contamination.

Steam cleaning and manual washing with normal pressure water are 2 possible methods of ensuring the efficacy of washing with high-pressure water in terms of reducing CNST contamination. In this study, we showed that both methods are useful in reducing the residual levels of CNST but that they cannot completely remove them. Steam cleaning, unlike washing with high-pressure water, does not scatter pieces of tissue, and therefore, it appears to be useful especially in removing spinal cord and dura mater fragments attached around the spinal column without scattering them. Helps *et al.* [4] reported that steam cleaning would contribute to dissemination of CNST fragments onto the external surface of carcasses by movement of the nozzle. During steam cleaning, the nozzle should not be moved from the internal surface to the external surface of the carcass and must be washed after cleaning of each carcass to prevent spread of CNST via the nozzle itself. As a preliminary washing procedure, washing with normal pressure water can be easily introduced without any special changes in the slaughtering line or installation of expensive equipment. Also, washing with normal pressure water is advantageous for preventing the spread of contamination because it scatters less water than manual washing with high-pressure water.

As shown in Fig. 2, manual washing with high-pressure water for 60 seconds can reduce even large amounts of residual CNST to nearly below the detection limit. However, the levels of CNST residue around the third cervical vertebra increased if only the upper part of the carcass was washed (Fig. 2B). This is probably due to CNST dripping down to the third cervical vertebra area when the carcasses were hung up by the hind legs. Therefore, in order to reduce CNST contamination below the detection level, it is important to perform washing in the correct order and for the appropriate duration. In addition, it is necessary to separate the washing area from the surroundings to prevent spread of CNST via the water if washing with high-pressure water is adopted.

It is important to understand the effect of the washing methods on CNST removal in order to choose a method that works properly. In the present study, we reported the actual situation of contamination of carcasses by CNST and showed the effects of different methods of carcass cleaning on CNST removal. Our results suggest an efficient method for removal of CNST contamination from the surface of carcasses. To minimize CNST contamination on the surface of carcasses, vacuum removal of the spinal cord is a prerequisite. High levels of CNST contamination, possibly caused by splitting, should be reduced by a preliminary washing step such as steam cleaning or washing with normal pressure water, and washing with high-pressure water should then be performed to reduce the CNST contamination to a negligible level. We believe that these results will provide useful information for creating sanitation standard operating procedures and thus contribute to reducing the risk of spread of BSE prion via carcasses and meat products contaminated with CNST.

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REFERENCES

1. Anil, M. H., Love, S., Helps, C. R. and Harbour, D. A. 2002. Potential for carcass contamination with brain tissue following stunning and slaughter in cattle and sheep. *Food Control* **13**: 431–436.
2. European Commission. 2002. Update of the opinion on TSE infectivity distribution in ruminant tissue. Available: http://ec.europa.eu/food/fs/sc/ssc/out296_en.pdf
3. EFSA. 2004. Report of the EFSA working group on BSE risk

from dissemination of brain particles in blood and carcass. *EFSA J.* **123** Available: http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620777397.htm

4. Helps, C. R., Hindell, P., Hillman, T. J., Fisher, A. V., Anil, H., Knight, A. C., Whyte, R. T., O'Niell, D. H., Knowles, T. G. and Harbour, D. A. 2002. Contamination of beef carcasses by spinal cord tissue during splitting. *Food Control* **13**: 417–423.
5. Iwata, N., Sato, Y., Higuchi, Y., Nohtomi, K., Nagata, N., Hasegawa, H., Tobiume, M., Nakamura, Y., Hagiwara, K., Furuoka, H., Horiuchi, M., Yamakawa, Y. and Sata, T. 2006. Distribution of PrP(Sc) in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in Japan. *Jpn. J. Infect. Dis.* **59**: 100–107.
6. Lim, D. G., Erwanto, Y. and Lee, M. 2007. Comparison of stunning methods in the dissemination of central nervous system tissue on the beef carcass surface. *Meat. Sci.* **75**: 622–627.
7. Masujin, K., Matthews, D., Wells, G. A., Mohri, S. and Yokoyama, T. 2007. Prions in the peripheral nerves of bovine spongiform encephalopathy-affected cattle. *J. Gen. Virol.* **88**: 1850–1858.
8. Prendergast, D. M., Sheridan, J. J., Daly, D. J., McDowell, D. A. and Blair, I. S. 2003. Dissemination of central nervous system tissue from the brain and spinal cord of cattle after captive bolt stunning and carcass splitting. *Meat. Sci.* **65**: 1201–1209.
9. Prendergast, D. M., Sheridan, J. J., Daly, D. J., McDowell, D. A. and Blair, I. S. 2004. Dissemination of central nervous system tissue during slaughter of cattle in three Irish abattoirs. *Vet. Rec.* **154**: 21–24.
10. Ramantanis, S. B. 2006. Alternative cattle slaughtering technologies and/or measures reducing the dissemination of central nervous system tissue during head handling, harvesting of cheek meat and tongue and carcass splitting-a review. *Vet. Arhiv.* **76**: 19–36.
11. Wells, G. A. H., Hawkins, S. A, Green, R. B., Austin, A. R., Dexter, I., Spencer, Y. I., Chaplin, M. J., Stack, M. J. and Dawson, M. 1998. Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Vet. Rec.* **142**: 103–106.
12. Wells, G. A., Spiropoulos, J., Hawkins, S.A. and Ryder, S. J. 2005. Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. *Vet. Rec.* **156**: 401–407.
13. Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P. G. 1996. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347**: 921–925.
14. Yeşilbag, K. and Kaikan, A. 2005. Detection of central nervous system tissues as BSE specified risk material in meat products in Turkey. *Food Control.* **16**: 11–13.

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Heat shock cognate protein 70 contributes to *Brucella* invasion into trophoblast giant cells that cause infectious abortion

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Abstract

Background: The cell tropism of *Brucella abortus*, a causative agent of brucellosis and facultative intracellular pathogen, in the placenta is thought to be a key event of infectious abortion, although the molecular mechanism for this is largely unknown. There is a higher degree of bacterial colonization in the placenta than in other organs and many bacteria are detected in trophoblast giant (TG) cells in the placenta. In the present study, we investigated mechanism of *B. abortus* invasion into TG cells.

Results: We observed internalization and intracellular growth of *B. abortus* in cultured TG cells. A monoclonal antibody that inhibits bacterial internalization was isolated and this reacted with heat shock cognate protein 70 (Hsc70). Depletion and over expression of Hsc70 in TG cells inhibited and promoted bacterial internalization, respectively. IFN-γ receptor was expressed in TG cells and IFN-γ treatment enhanced the uptake of bacteria by TG cells. Administering the anti-Hsc70 antibody to pregnant mice served to prevent infectious abortion.

Conclusion: *B. abortus* infection of TG cells in placenta is mediated by Hsc70, and that such infection leads to infectious abortion.

Background

Brucellosis is a widespread and economically important infectious disease of animals and humans caused by members of the genus *Brucella*. *Brucella* spp. are small gram-negative, facultative intracellular pathogens that cause abortion, retained placenta and infertility in numerous domestic and wild mammals, and a disease known as undulant fever in humans [1-3]. Transmission of *Brucella* spp. from infected animals to humans may be either direct or indirect. Direct transmission involves the respiratory, conjunctival and cutaneous routes, and is more important in people in close contact with infected animals. Indirect

transmission is through the consumption of contaminated dairy products [3]. *Brucella* spp. occasionally causes spontaneous abortion in pregnant women [4].

There have been several histological studies on the placentas of *Brucella* infected animals [5]. Further, it has been found that *Brucella* internalizes into the caprine erythrophagocytic trophoblastic epithelial cells from the maternal circulation [6] and that the internalized bacteria replicate within the rough endoplasmic reticulum, resulting in secondary infection of adjacent trophoblastic epithelial cells [6,7]. Researches have also shown that after necrosis of infected trophoblasts, large numbers of brucellae are released, and proximity of the fetal capillaries in the ulcerated placenta to the lumenal bacteria has been proposed as the source of the fetal bacteremia and further placental infection [6,8]. However, the molecular mechanism of abortion induced by *Brucella* spp. remains unknown.

The mouse model, particularly that using the unpregnant mouse, has been used extensively to study some aspects of the pathogenesis of brucellosis [2]. While brucellosis is known to primarily affect the reproductive tract in the natural host, little is known regarding the cellular and molecular mechanisms of *Brucella* infection in the pregnant mouse [9]. Although the structure of bovine placenta is completely different from mouse placenta, the infectious abortion model using the pregnant mouse is a powerful tool for investigating the mechanisms of *Brucella* pathogenesis. In our previous study, we demonstrated that *B. abortus* causes abortion in pregnant mice by inoculating bacteria on day 4.5 of gestation [10]. We found that there was a higher degree of bacterial colonization in the placenta than in other organs, that there were many bacteria in trophoblast giant (TG) cells in the placenta and that an intracellular replication-defective mutant did not induce abortion. These findings suggest that bacterial infection of TG cells plays a key role in abortion induced by *B. abortus* infection.

Pregnancy leads to a generalized suppression of the adaptive immune system, typified by significantly decreased cell-mediated immunity and reduced T helper cell (Th) 1 responsiveness [11-13]. This immunosuppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents [14,15]. Our previous study showed that a transient increase in interferon (IFN)-γ due to *Brucella* infection contributes to abortion in pregnant mice [10]. In addition to examining the balance of inflammatory and regulatory cytokines in bacteria infected pregnant mice, analysis of bacterial internalization into the TG cells, a specific host cells in placenta, will help to advance our knowledge regarding the control of *Brucella*-induced abortion.

In the present study, we investigated the internalization of *B. abortus* into TG cells and identified heat shock cognate protein 70 (Hsc70) as a candidate receptor against *Brucella* or bacterial uptake-associated molecule. We noted that IFN-γ enhances bacterial internalization into TG cells.

Methods

Bacterial strains

All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abortus* biovar 1 strains. GFP expressed 544 strain was used in this study [16,17]. *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% agar.

Mice

Six to ten-week-old ICR female mice were individually mated to 6- to 10-week-old ICR male mice. The parent mice were obtained from CLEA Japan. Day 0.5 of gestation was the day the vaginal plug was observed. The normal gestational time for these mice is 19 days.

Virulence in pregnant mice

Groups of five pregnant mice were infected intraperitoneally with approximately 10^4 colony forming unit (CFU) of brucellae in 0.1 ml saline on day 4.5 of gestation [10]. On day 18.5 of gestation, placenta and spleen were removed and homogenized in phosphate buffered saline (PBS). Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFU in each organ. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat. The animal experiments were permitted by Animal Research Committee of Obihiro University of Agriculture and Veterinary Medicine.

Cell culture

Trophoblast stem (TS) cells were cultured in TS medium in the presence of FGF4, heparin and mouse embryonic fibroblast (MEFs)-conditioned medium as described previously [18]. The TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640. To induce differentiation to trophoblast giant (TG) cells, the cells were cultured in the only TS medium alone for 3 days at 37°C in CO_2 incubator. The TG cells were seeded ($1–2 \times 10^5$ per well) in 48 well tissue culture plates for all assays.

Efficiency of bacterial internalization and replication within cultured cells

Bacterial infection and intracellular survival assays were performed according to a modified version of the method of Kim *et al* [19]. *B. abortus* strains were deposited onto TS or TG cells at a multiplicity of infection (MOI) of 100 which had been grown on 48-well microtiter plates containing TS medium but no antibiotics by centrifugation at 150 × g for 10 min at room temperature. To measure bacterial internalization efficiency after 30 min of incubation at 37°C, the cells were washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 30 min. Next, cells were washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on Brucella plates. To measure intracellular replication efficiency, infected cells were incubated at 37°C for 30 min, washed once with TS medium and then incubated with TS medium containing gentamicin (30 μg/ml) for 2, 24, 48 and 72 h. The cell washing, lysis and plating procedures were the same as for the bacterial internalization efficiency assay. Percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum. The purified the R2–25 antibody or recombinant IFN-γ (Cedarlane Laboratories) was added to the TS medium at the indicated concentrations 2 or 12 h before infection.

F-actin staining

GFP-expressing bacteria were deposited onto the cultured cells by centrifugation and the incubation was conducted at 37°C for 30 min. The infected cells were incubated with TS medium containing gentamicin (30 μg/ml) at 37°C for 30 min to kill extracellular bacteria and were then fixed in 4% paraformaldehyde for 30 min at room temperature. Next, samples were permeabilized in 0.2% Triton X-100, washed three times with PBS and incubated with Alexa Fluor 594-phalloidin (Molecular Probes) at 20 μg/ml for 30 min at 37°C. After three washes with PBS, samples were placed in mounting medium (90% glycerol containing 1 mg/ml phenylenediamene in PBS, pH 9.0) and visualized by fluorescence microscopy.

Isolation of monoclonal antibodies

Hybridomas producing monoclonal antibodies that inhibit bacterial internalization into TG cells were obtained from fusions of BALB/c P3-X63-Ag8.653 (8-azaguanine-resistant and non-producer cell line) myeloma cells with spleen cells from Wister rats that had been immunized with TG cells. The screening of hybridoma supernatants for inhibiting antibodies was performed by adding antibodies to the TS medium in a bacterial internalization assay. Monoclonal antibodies obtained from hybridoma supernatants were purified using a protein G column (GE Healthcare Life Science) and the class and subclass of the purified monoclonal antibodies were determined using an Immunogloblin Typing Kit (WAKO Pure Chemical). The R2–25 monoclonal antibody used in this study was typed as IgG1.

Subcellular fractionation of TS and TG cells

TS and TG cells (3×10^5/ml) were seeded into each well of a 6-well plate. Protein isolation for the cytoskeleton, nuclear, membrane, and cytosol fraction was performed using a ProteoExtract Subcellular Proteome Extraction Kit as described by the manufacturer (Calbiochem).

Immunoblotting

The cell lysates (500 μg/ml) and fractionated proteins (50 μg/ml) were separated on 10% polyacrylamide gels and transferred to a PVDF membrane, which was incubated for 1 h at room temperature with primary antibody (0.5 μg/ml) in 5% skim milk. It was then washed three times in Tris buffered saline (TBS) with 0.02% Tween 20, incubated for 30 min with a horseradish peroxidase (HRP)-conjugated secondary antibody at 0.01 μg/ml and then washed again. Immunoreactions were visualized by ECL (GE Healthcare Life Science). Antibodies for β-actin, β-tublin and histone H1 were purchased from SIGMA or Abcam. Anti-IFN-γ receptor rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology.

Mass spectrometry analysis

Identification of proteins reacting with monoclonal antibodies that inhibited bacterial internalization into TG cells was conducted by means of nano LC-MS/MS analysis and a search of MASCOT database (APRO life Science Institute, Japan).

RNA isolation and RT-PCR

The total RNA of TG cells was isolated using an RNA Purification Kit (Qiagen) and purified RNA samples were stored at -30°C until use. The RNA was quantified by absorption at 260 nm using a SmartSpec3000 spectrophotometer (Bio-Rad). RT-PCR was carried out using a Sperscript II Kit (Invitrogen). The primers used for mouse Hsc70 or β-actin amplification had the following sequence 5'-GCAGCTGGGCCTACACACAAG-3' and 5'-CCCTGTGGAACAAAGCTACAC-3', or 5'-CGTGACATTAAGGAGAAGCTGTGC-3' and 5'-CTCAGGAGGAGCAATGATCTTGAT.

Expression and purification of recombinant proteins

Mouse Hsc70 cDNA (GenBank Accession No. BC066191) was amplified from RNA isolated from TG cells by means of RT-PCR with the pair of primers described above. The product was cloned into the pCR2.1-TOPO vector (Invitrogen) (pCR-Hsc70). To achieve expression of recombinant Hsc70 protein, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into pCold TF vector

(Takara Bio Inc.). The His-tagged Hsc70 was expressed in the *E. coli* strain DH5α, and its purification and cleavage of His-tagged by HRV 3C protease were performed as described by the manufacturer (Novagen). Bovine Hsc70 and the rat anti-Hsc70 monoclonal antibody (SPA-815) were obtained from Stressgen for use as control materials.

To achieve expression of Hsc70 in TG cells, amplified DNA encoding Hsc70 from pCR-Hsc70 in PCR was cloned into the pcDNA4/TO vector in the T-Rex System (Invitrogen). pcDNA4/TO-Hsc70 was transfected into TG cells using the FuGENE 6 Transfection Reagent (Roche) with a final concentration of 1.2 μg/ml.

siRNA experiment

The siRNA duplexes used for silencing mouse Hsc70 (target sequence: AACAAGTAACATGGAATAATA), and β-actin (target sequence: CACTGACTTGAGACCAATAAA) and AllStars Negative Control siRNA were purchased from QIGEN. TG cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 10 nM for siRNAs.

Immunofluorescence microscopy

Samples grown on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with or without 0.2% Triton X-100 in PBS for 20 min at room temperature. After blocking with 5% BSA in PBS, the cells were incubated with primary antibody (25 μg/ml) for 1 h at 37°C, and detection was conducted with TRITC-labeled goat anti-rat IgG (0.01 μg/ml) (Chemicon). Fluorescent images were taken using an Olympus BX51 microscope and a cooled CCD camera Olympus DP70.

In vivo *depletion of Hsc70*

Hsc70 was neutralized in the mice by administering an anti-mouse Hsc70 monoclonal antibody (clone R2–25)*in vivo* using 100 or 200 μg of antibody in a volume of 0.3 or 0.6 ml intraperitoneally 24 h before infection. Control mice were given 100 μg of normal rat IgG in a volume of 0.1 ml according to the same injection schedule as used for the anti-Hsc70 monoclonal antibody treated groups. Bacterial infection was conducted as described previously. On day 18.5 of gestation, fetuses were removed from the mice and a judgment made as to whether they were pregnant or not. Fetuses were determined to be alive if there was a heartbeat, and dead if there was no heartbeat.

Statistical analysis

All statistical analysis was conducted using the Student *t* test.

Results

B. abortus *internalizes and replicates in trophoblast giant cells*

We previously reported that there were many bacteria in trophoblast giant (TG) cells in the placenta by inoculation of pregnant mice with *B. abortus* [10]. To examine this bacterial infection into TG cells further, we used *in vitro* cell culture system of trophoblast stem (TS) cells and TG cells differentiated from TS cells. The *B. abortus* internalized into TG cells more efficiently than TS cells (Fig. 1A). We also investigated the intracellular replication of *B. abortus* in TS and TG cells. The bacteria replicated more efficiently in TG cells than TS cells (Fig. 1B).

Several intracellular pathogens attached to the host plasma membrane induce actin polymerization around the site of bacterial attachment and the process is essential for bacterial entry [20]. We therefore examined actin polymerization by means of fluorescence microscopy after 30 min and 48 h of incubation of TS and TG cells infected with *B. abortus*. It has been noted that differentiated TG cells dramatically rearrange their actin cytoskeleton into thick bundles of stress fibers [21]. There was no apparent actin polymerization around the site of the bacterial entry after 30 min incubation on TG cells or 48 h of incubation of infected TG cells (Fig. 1C).

Isolation of monoclonal antibodies that inhibit bacterial internalization into TG cells

To identify a receptor against *B. abortus* on TG cells, rats were immunized with TG cells, and monoclonal antibodies that inhibit bacterial internalization into TG cells were isolated. Seventy-five clones of bacterial internalization inhibiting antibodies (3.9%) were isolated from 1,920 hybridoma supernatants. From among the monoclonal antibodies, we selected R2–25, since it significantly inhibited internalization and showed clear reactions with protein in immunoblotting. The purified R2–25 antibody significantly inhibited bacterial internalization concentration dependently, but there was no inhibition with rat IgG (negative control) (Fig. 2A). The R2–25 antibody reacted with protein of around 70 kDa which was localized in membrane and cytosol, with the protein amount in the membrane fraction being especially large (Fig. 2C). On examining the distribution of protein reacting with R2–25 on the surface of TG cells by immunofluorescence microscopy, we observed an islet-like distribution on the surface of TG cells which was no permeabilized cells (Fig. 2B). It was difficult to detect the protein reacting with the R2–25 antibody on the surface of permeabilized cells.

Antibody inhibiting bacterial internalization reacts with heat shock cognate protein 70

We performed mass spectrometry analysis to identify the protein reacting with the R2–25 antibody. Proteins of the

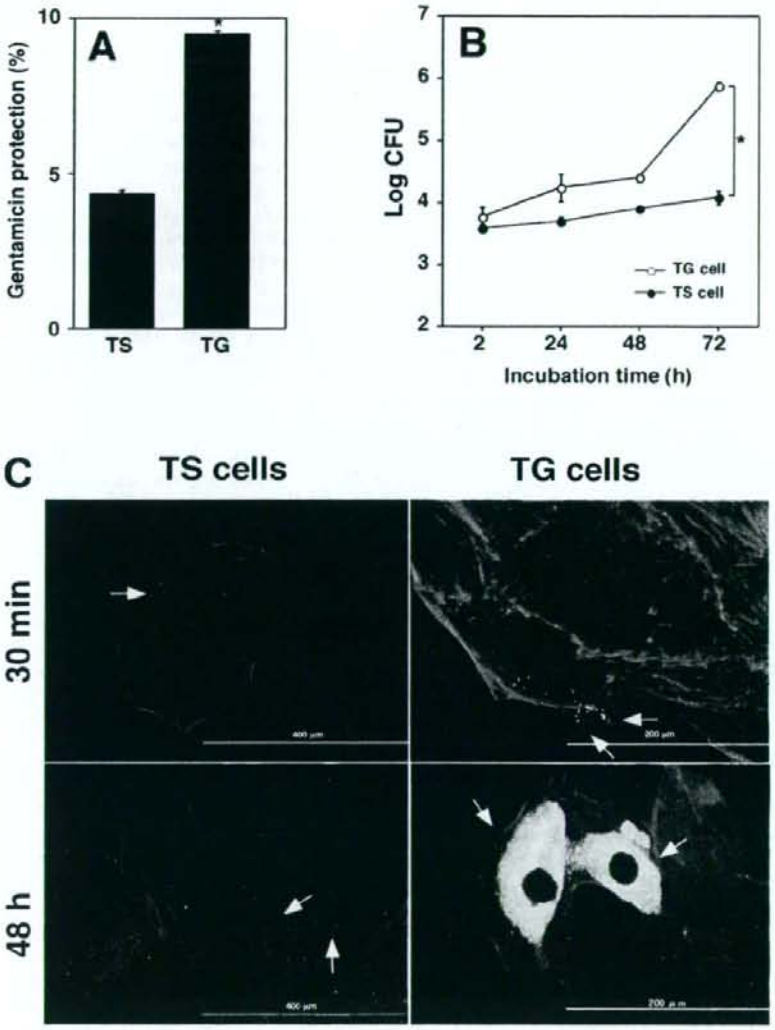


Figure 1

***B. abortus* infection in trophoblast giant cells.** (A) Bacterial internalization into trophoblast stem (TS) and giant (TG) cells. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant difference between the bacterial internalization into TS and TG cells is indicated by asterisk (*, $P < 0.01$). (B) Intracellular replication of *B. abortus* in TS and TG cells. Datum points and error bars represent the mean of CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviations. Statistically significant difference between bacterial replication of *B. abortus* in TS and TG cells after 72 h of inoculation is indicated by asterisk (*, $P < 0.01$). (C) F-actin staining of bacteria infected cells. The figure shows GFP (bacteria) and Alexa Fluor 594 (actin filaments) channel merged images. Arrows indicate TS and TG cells containing bacteria.

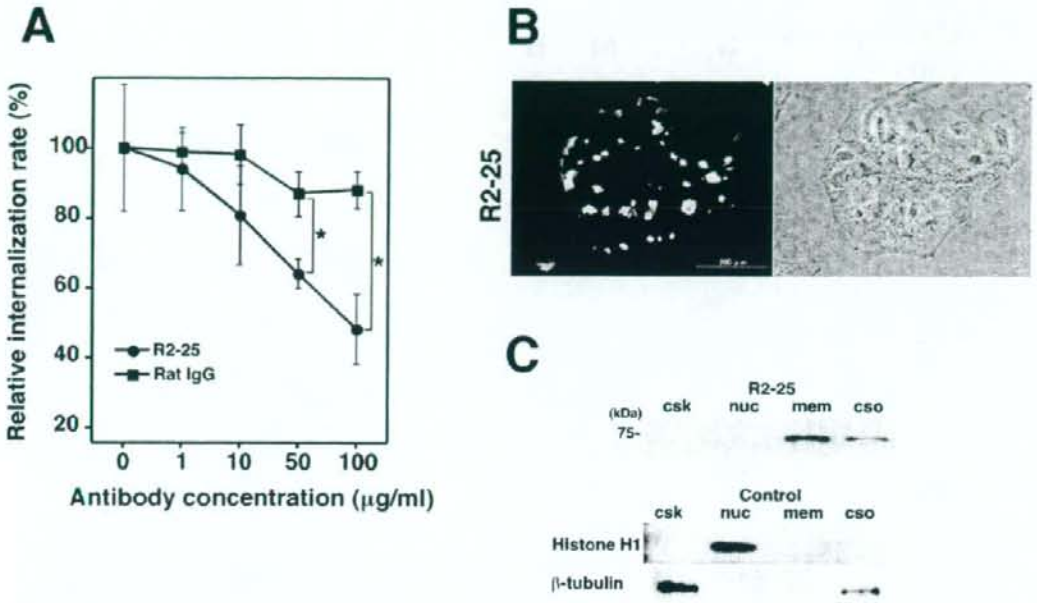


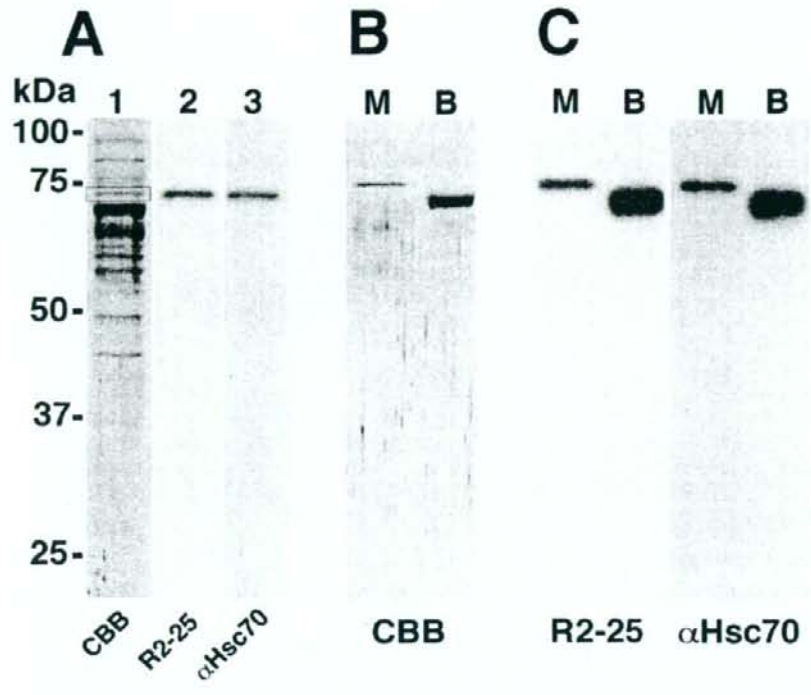
Figure 2

Characterization of the monoclonal antibody that inhibits bacterial internalization into TG cells. (A) Inhibition of bacterial internalization by the R2–25 antibody treatment. 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 treated with the R2–25 antibody and those treated with rat IgG are indicated by asterisks (*, $P < 0.01$). (B) Distribution of protein reacting with monoclonal antibodies in TG cells. The left panels show fluorescence microscopy of the antibody stained TG cells and the right panels phase contrast microscopy of the corresponding microscopic fields. (C) Immunoblot analysis was performed on TG cell subcellular fractions with the monoclonal antibodies R2–25. Cells were fractionated to cytoskeleton (csk), nucleus (nuc), membrane (mem) and cytosol (cso). The anti-histone H1 and anti-β-tubulin antibody were used for fraction control for the nucleus and cytoskeleton.

membrane fraction were separated by SDS-PAGE and transferred to a PVDF membrane (Fig. 3A). The protein reacting with R2–25 was extracted from the PVDF membrane and the subjected to LC-MS/MS analysis. Through a search of the MASCOT database, the protein reacting with R2–25 was determined to be heat shock cognate protein 70 (Hsc70) (Fig. 3D). The anti-Hsc70 antibody also reacted with this protein (Fig. 3A). To confirm that the R2–25 antibody reacted with Hsc70, we tested its reactivity with mouse and bovine recombinant Hsc70. As expected, the R2–25 and anti-Hsc70 antibodies reacted with both types of recombinant Hsc70 (Fig. 3B and 3C). The mouse recombinant Hsc70 had a slightly greater molecular weight than the bovine recombinant Hsc70 because 25 amino acid residues had been added to the former after HRV 3C protease cleavage. These results indicate that the protein reacting with R2–25 was Hsc70.

Hsc70 contributes to bacterial internalization into TG cells

To examine the effect of Hsc70 on bacterial internalization into TG cells further, we reduced the amount of endogenous Hsc70 by transfecting Hsc70-specific small interfering RNA (siRNA) duplexes into the TG cells. After 48 h of transfection with Hsc70-specific siRNA, the expression level of Hsc70 was no longer detectable, but was not affected by transfection with β-actin or the control siRNA (Fig. 4A and 4D). Thus, the internalization efficiency of *B. abortus* into TG cells was significantly reduced by transfection with Hsc70-specific siRNA (Fig. 4C). Next, excessive production of endogenous Hsc70 was induced



D

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  1 MSKGPAVGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
 51 IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVVNDAGR
101 PKVQVEYKGE TKSFYPEEVS SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF
151 NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL
201 GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK
251 KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 RFEELNADLF RGTLDPVEKA LRDAKLDKSQ IHDIVLVGGS TRIPKIQKLL
351 QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS
401 LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
451 KDNNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
501 ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQRDKVSSKN SLESYAFNMK
551 ATVEDEKLQG KINDEDKQKI LDKCNEIISW LDKNQTAEEE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD
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Figure 3

Bacterial internalization inhibiting antibodies react with Hsc70. (A) A membrane to which TG cell membrane fraction proteins had been transferred was stained with Coomassie brilliant blue (CBB) (lane 1), immunoblotted with the R2–25 antibody (lane 2) and the anti-Hsc70 antibody (clone SPA-815) (lane 3). (B) CBB staining of membrane to which mouse (M) or bovine (B) recombinant Hsc70 had been transferred. (C) Immunoblot analysis of mouse (M) or bovine (B) recombinant Hsc70 with R2–25 antibody and anti-Hsc70 antibody (clone SPA-815). (D) Amino acid sequences of Hsc70. The protein reacting with R2–25 was extracted from the PVDF membrane (square in lane 1 of panel A) and was used for LC-MS/MS analysis. The amino acids detected for Hsc70 in the analysis are shown in bold.

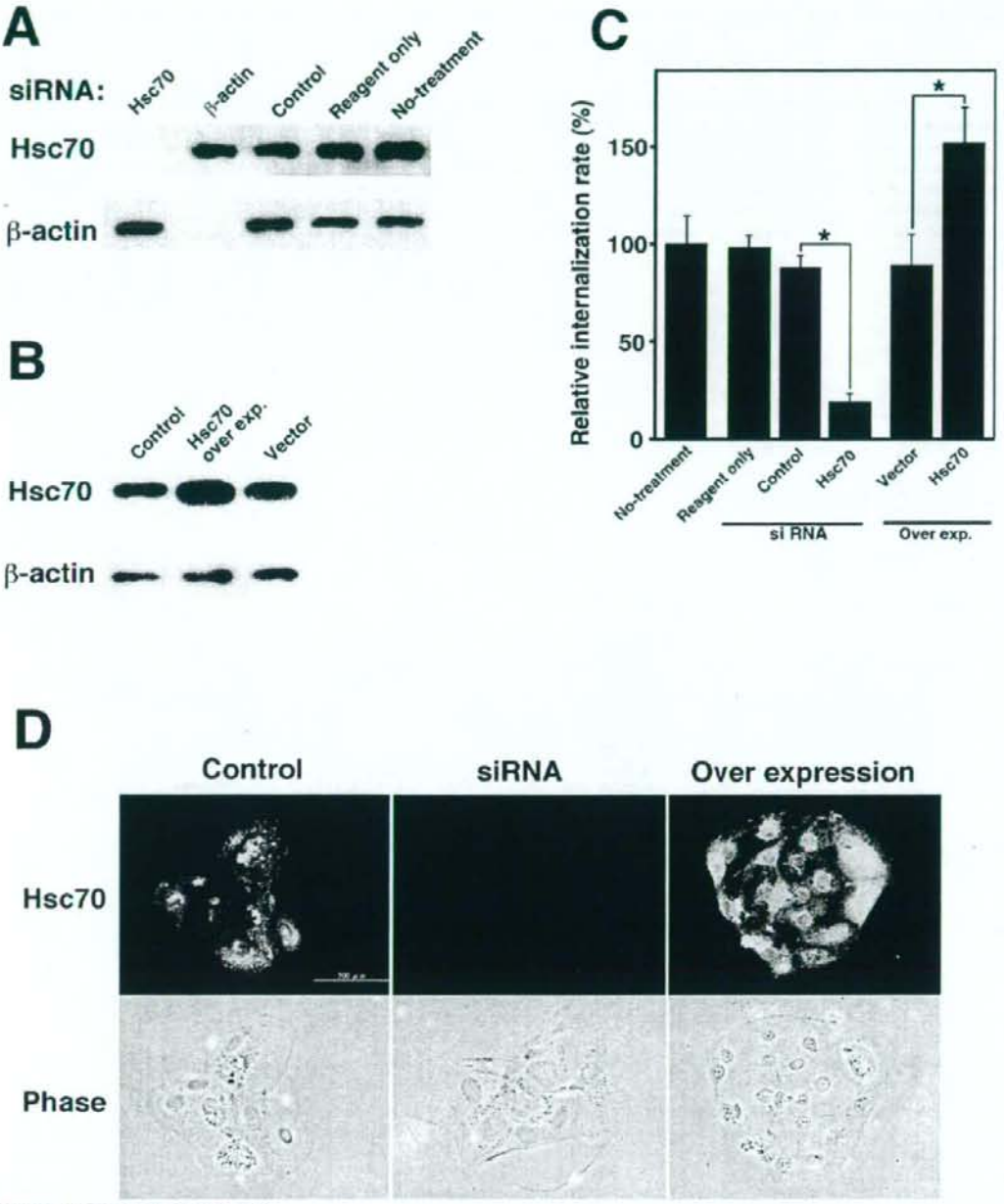

Figure 4 *(see legend on next page)*