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Short communication

Molecular cloning and sequencing of the cDNA encoding the bat CD4

Tsutomu Omatsu [a,*], Yorihiro Nishimura [b], Eun Jung Bak [a], Yoshiyuki Ishii [a], Yukinobu Tohya [b], Shigeru Kyuwa [a], Hiroomi Akashi [b], Yasuhiro Yoshikawa [a]

[a] *Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

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Abstract

Chiroptera is thought to be a vector or a natural reservoir of various pathogenic microbes. However, there are few basic studies on the subject of chiroptera immune systems. This is the first report to determine the sequence of bat CD4 cDNA. Comparison with other animals' CD4 and phylogenetic analysis have shown that bat CD4 had a higher homology to cat and dog CD4 than to human and mouse CD4. Moreover, from the analysis of the structure of the CD4 Ig-like C-type 1 region, in bat CD4 there was an insertion of 18 extra amino acids. In addition, bat CD4 lacked cystein, which suggested that the disulfide bond could not be formed. Human, monkey and mouse CD4 have the cystein and the disulfide bond, but pig, cat, whale and dog CD4, like that of the bat, lacked the cystein. We conducted the present study in order to help elucidate the infectious diseases derived from the bat as well as bat immune systems.

Keywords: Bat; CD4; cDNA; Cloning; Ig-like C-type 1 region

1. Introduction

Chiroptera is the natural reservoir of rabies, Nipah virus, European bat lyssavirus types 1 and 2, and Australian bat lyssavirus (Chua et al., 2002; Field et al., 2001; Hanna et al., 2000; Johnson et al., 2002; Messenger et al., 2002). In addition, chiroptera may be a vector of other emerging and re-emerging infectious diseases (Field et al., 2004; Pilipski et al., 2004; Sulkin et al., 1966). Nevertheless, few basic immunological studies on chiroptera have been done.

The antigen recognition of T cells requires interaction between TCR and MHC molecules. CD4 and CD8 molecules are the surface proteins engaged in signal regulation during these interactions. The surfaces of mature T cells expressed either CD4 or

* Corresponding author. Tel.: +81 3 5841 5037; fax: +81 3 5841 8186.
E-mail address: aa37157@mail.ecc.u-tokyo.ac.jp (T. Omatsu).

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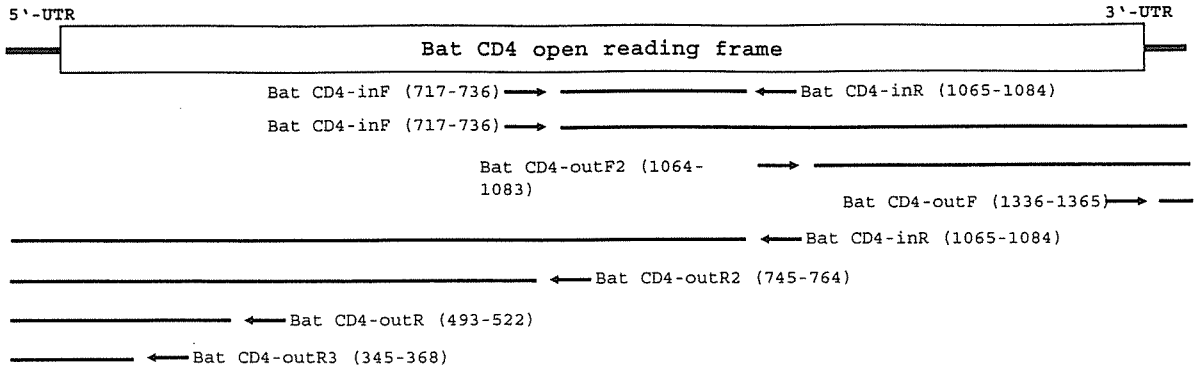

Fig. 1. Schematic structure of the bat CD4 cDNA and the cloning strategies by PCR. The locations and directions of new PCR primers are indicated by arrows. The numbers shown in parentheses indicate the position of each primer in Fig. 2.

CD8 (Thomas et al., 1984; Veillette et al., 1988a). CD4-expressing T cells react to the antigen presented by the MHC classII molecule (Swain et al., 1984). CD4 protein is a glycoprotein of an adherent molecule group belonging to the immunoglobin superfamily. It has four Ig-like regions in the extracellular part and no intracellular enzyme active site; CD4 connects to tyrosine kinase, Lck, belonging to the *Src* family specific for lymph cells (Veillette et al., 1988b). In this study, we determined the sequence of CD4 including the full open reading frame, and analyzed the bat CD4 from both phylogenetic and comparative zoological viewpoints.

2. Materials and methods

Egyptian rousettes (*Rousettus aegyptiacus*) were purchased from the Asada Choujuu Boueki Co., Ltd., Japan. Fresh spleen sample was collected from Egyptian rousettes under anesthesia with ketamine and preserved by freezing at −80 °C until use. Total RNA was isolated from the bat spleen with ISOGEN (NIPPON GENE CO., Ltd.), and a cDNA library was subsequently constructed using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). The Marathon cDNA libraries were used as a template for the PCR reaction.

5′ primer (5′-CTCCCTACTTAACTTTGAAG-3′) and 3′ primer (5′-CAGTTTGGTTCTC TGGCTTC-3′) were designed from the sequence data of cat, dog and pig CD4. Bat CD4 was amplified with these pri mers by polymerase chain reaction (PCR). The PCR product was isolated by electrophoresis in a 1% agarose gel (Wako) and purified with QIAquick Gel Extraction Kit (QIAGEN). The purified sample was cloned with TOPO TA cloning kit (Invitrogen) and cycle sequenced with Big Dye™ terminator (ABI). The sequence was determined with ABI PRISM™ 377 DNA Sequencer (ABI). We designed new primers from this sequence data and amplified the entire remaining cDNA of CD4 by semi-nested 5′- and 3′-rapid amplification of cDNA ends (RACE). The position of each new primer and cloning strategies by PCR are shown in Fig. 1.

CD4 amino acid sequence data of other animals were obtained from the Gene Bank. Sequence data were aligned using Clustal W (Higgins et al., 1996) and checked by eye, and all positions with gaps or ambiguous alignment were excluded from the analysis. A phylogenetic tree was constructed using MEGA 2 (Kumar et al., 2001).

This experiment was performed in accordance with the Animal Experimentation Guidelines of the University of Tokyo, and approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

3. Results and discussion

We determined the nucleotide sequence of bat cDNA including the full-length open reading frame of CD4. The nucleotide sequence and amino acid sequence of bat CD4 are shown in Fig. 2. The

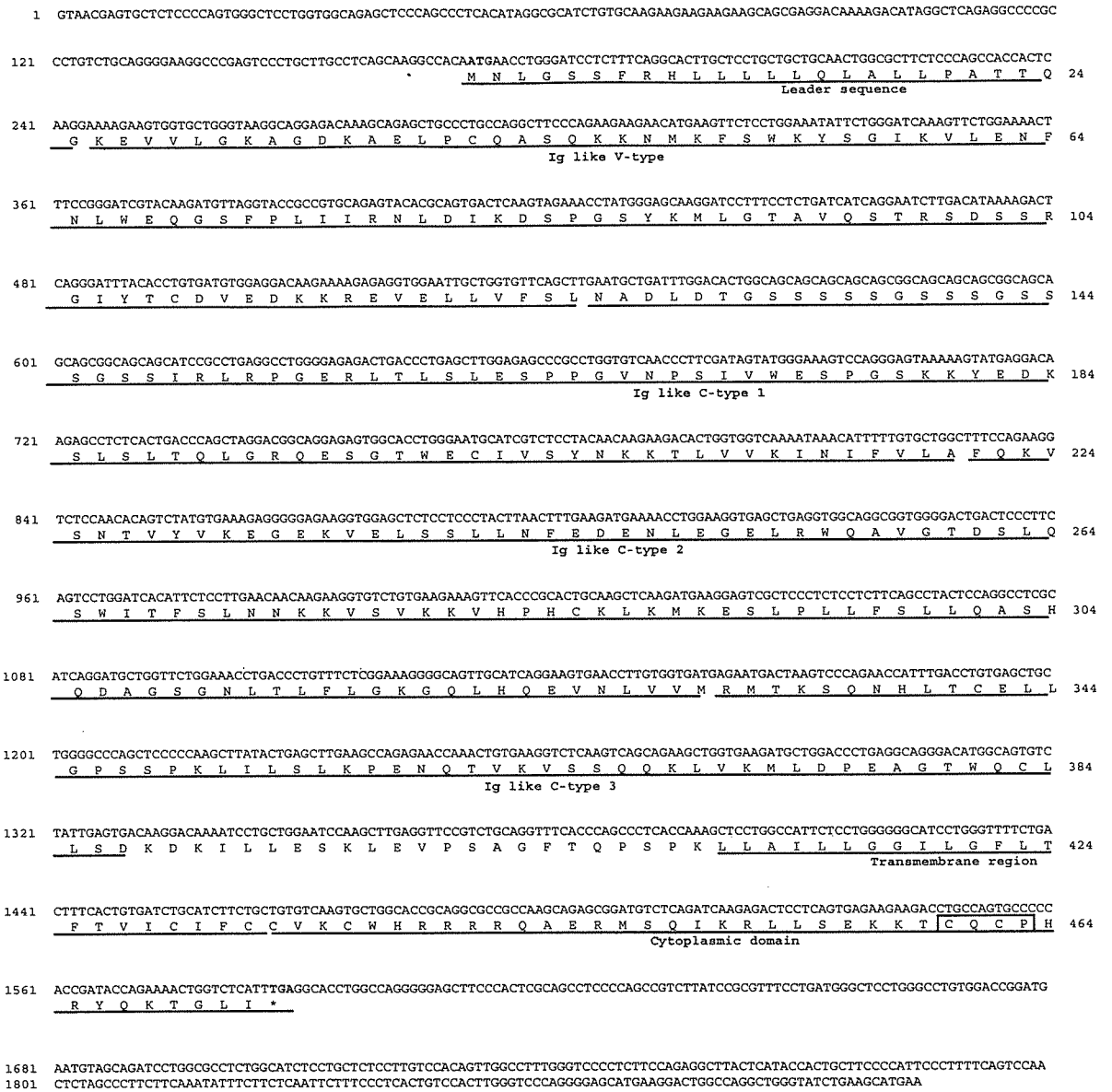

Fig. 2. The nucleotide sequence of bat CD4 (accession no. AB210837) and the predicted amino acid sequence. The left-side numbers indicate the nucleotide position. The right-side numbers indicate the amino acid position. Amino acid residues are shown by the one-letter abbreviation code below the nucleotide sequence. Nucleotides in the 5′ and 3′ non-coding regions are shown preceding the ATG (methionine codon) and following the TGA or termination codon, indicated by *. The locations of the CD4 structures are indicated with names and solid lines under the corresponding amino acid sequences. The consensus motif (-C-Q-C-P-) found to be associated with $p56^{lck}$ is boxed.

sequenced cDNA included a total of 1905 nucleotides, in which 1419 nucleotides encoded 472 amino acids. The homology of the nucleotide and amino acid sequences between the bat and other animals is shown in Table 1. On both the nucleotide and amino acid sequences, bat CD4 had a higher homology to both the cat and dog CD4 than to the CD4 of other animals such as humans and mice.

The phylogenetic tree with the amino acid sequence of CD4 is shown in Fig. 3. The results

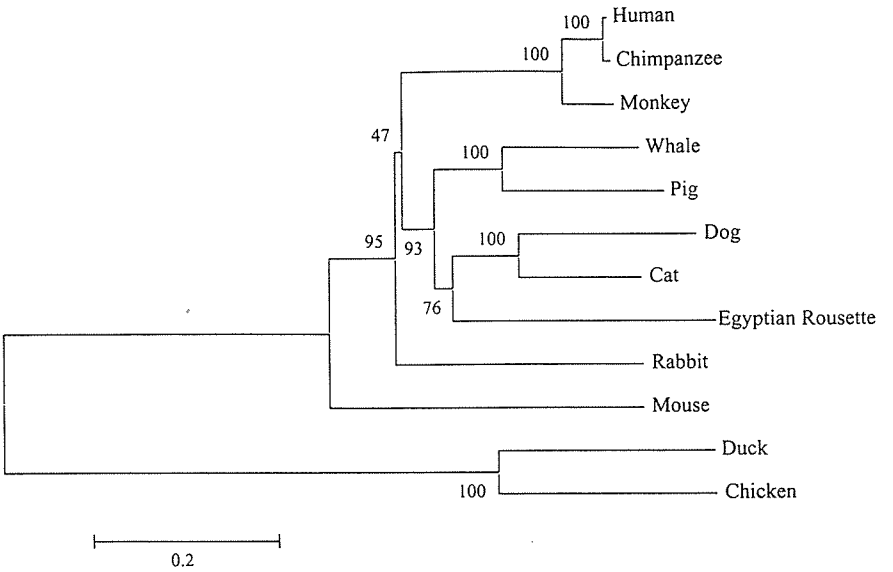

Fig. 3. Neighbor-joining tree constructed by MEGA 2 using the amino acid sequence of CD4 of the bat, human (accession no. AAV38614), chimpanzee (NP_001009043), monkey (P79184), whale (AAD23738), pig (NP_001001908), dog (NP_001003252), cat (NP_001009250), rabbit (P46630), mouse (NP_038516), duck (AAK59279), and chicken (AAS67022). The numbers at the nodes indicate the bootstrap values for the NJ method. The scale bar corresponds to 20% sequence divergence.

showed that bat CD4 was closely related to cat and dog CD4 and was distant from duck and chicken which agrees with the results shown in Table 1. These results supported the phylogenetic analysis of mitochondrial DNA (Lin and Penny, 2001; Nikaido et al., 2001). The amino acid sequence of the CD4 Ig-like C-type 1 region was compared between the bat and other animals (Fig. 4). In bat CD4, there was an insertion of

```
                                                                ▼
Human      1:TANSDT-----------------HLLQGQS-LTLTLESPPGSS-PSVQCRSPRGKNIQG 40
Monkey     1:TANSDT-----------------HLLEGQS-LTLTLESPPGSS-PSVKCRSPGGKNIQG 40
Mouse      1:TFSPGT-----------------SLLQGQS-LTLTLDSNSKVSNPLTECKHK-KGKVVS 40

                                                               ▽
Rousette   1:NADLDTGSSSSSGSSSGSSSGSSIRLRP-GERLTLSLESPPGVN-PSIVWESPGSKKYED 58
Cat        1:TAKVDPSGSGGSSSS-STST-STSIYLLQGQSLTLTLESPSSSN-PSVQWKGPGNKSKSG 57
Dog        1:TAKWDSGSSS---------GSSNIRLLQGQQ-LTLTLENPSGSS-PSVQWKGPGNKSKHG 49
Rabbit     1:TANPNT-----------------RLLHGQS-LTLTLEGPSVGS-PSVQWKSPENKIIET 40
Pig        1:LTASVT-----------------RVLL-GQSLTLTLEGPSGSH-PTVQWKGPGNKSKND 40

                                    ▼
Human     41:GKT-LSVSQLELQDSGTWTCTVLQN-QKKVEFKIDIVVLA                    78
Monkey    41:GRT-ISVPQLERQDSGTWTCTVSQD-QKTVEFKIDIVVLA                    78
Mouse     41:GSKVLSMSNLRVQDSDFWNCTVTLD-QKKNWFGMTLSVLG                    79

                                    ▼
Rousette  59:-KS-LSLTQLGRQESGTWECIVSYN-KKTLVVKINIFVLA                    95
Cat       58:VHS-LSLSQLELQESGTCTCTVSQS-QKTLVFNTNILVLA                    95
Dog       50:GQN-LSLSWPELQDGGTWTCIISQS-QKTVEFNINVLVLA                    87
Rabbit    41:GPT-CSMPKLRLQDSGTWSCHLSFQDQNKLELDIKIIVLG                    79
Pig       41:VKS-LLLPQVGLEDSGLWTCTVSQD-QKTLVFRSNIFVLA                    78
```

Fig. 4. Comparison of the CD4 Ig-like C region between the bat and human, monkey mouse, cat, pig, dog and whale. Identical amino acid residues are indicated by dots (·) and gaps are indicated by bars (–). The cystein residues consisting of the disurfide bond are indicated by closed triangles. The residues in which tryptophan is substituted for cystein are indicated by open triangles.

Table 1
Homology of CD4 nucleotide and amino acid sequence between bat and other species

	Nucleotide (%)	Amino acid (%)
Human	57.9	56.4
Mouse	61.0	47.6
Cat	75.5	60.6
Dog	73.0	59.4
Chicken	46.9	23.5

18 extra amino acids in the beginning of this region, where dog and cat CD4 have 9 and 16 amino acids insertions, respectively. The insertion might influence the relationship between V-type region and C-type 1 region. Furthermore, the N-terminus side of the cystein pair was replaced with tryptophan, whereas in the case of human, monkey and mouse CD4 it is cystein. These latter animals have a disulfide bond formed by the two cysteins in this region. However, bat CD4 lacked the disulfide bond, as in the cat, dog and whale, and the conformation of bat CD4 Ig-like C-type 1 region might be different from that of the human and mouse. The conformational change of the bat CD4 might have influenced the antigen presentation process between dendritic cells and helper T cells by means of CD4-MHC classII connection and T cell activity (Milde et al., 1993; Norimine et al., 1992).

There have been few studies of the immune systems of the bat, which has been thought to be a vector or a natural host of various pathogenic microbes. We believe that the present study will help elucidate the infectious diseases derived from the bat and further our understanding of bat immunology.

Acknowledgements

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Roles of the small intestine for induction of toll-like receptor 4-mediated innate resistance in naturally acquired murine toxoplasmosis

Takahisa Furuta[1], Takane Kikuchi[1,5], Shizuo Akira[2], Naohiro Watanabe[3] and Yasuhiro Yoshikawa[4]

[1]Division of Infectious Genetics, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo 108-8639, Japan
[2]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 665-0871, Japan
[3]Department of Tropical Medicine, Jikei University School of Medicine, Tokyo 105-8461, Japan
[4]Department of Biomedical Science, Graduate School of Agricultural and Life Science, University of Tokyo, Tokyo 113-8657, Japan

[5]Present address: Department of Veterinary Biosciences, College of Veterinary Medicine, Ohio State University, 1925 Coffey Road, Columbus, OH 43210-1093, USA

Keywords: innate immunity, peroral infection, toll-like receptor 4, toxoplasmosis

Abstract

Peroral infection of *Toxoplasma gondii* is thought to reflect the typical infection route of naturally acquired toxoplasmosis in humans. We have investigated possible differential roles of toll-like receptor 2 (TLR2) and TLR4 in host defense against naturally acquired murine toxoplasmosis. After peroral inoculation of *T. gondii* ME49 cysts, TLR4-deficient C3H/HeJ mice were more susceptible to infection than wild-type (WT) C3H/HeN mice, as shown by increased cyst number and low production of cytokines, which are the key factors in protective immunity. When mice were inoculated by intra-peritoneal inoculation of *T. gondii*, there were no significant differences in the number of brain cysts and cytokine productions between C3H/HeJ and C3H/HeN mice. Histopathologic examination revealed severe inflammation in the small intestine of C3H/HeJ (TLR4-deficient) mice, while an increased number of TLR4-positive mononuclear cells was found in C3H/HeN (WT) mice. To confirm these phenomena, $TLR2^{-/-}$ or $TLR4^{-/-}$ mice were infected perorally with *T. gondii* cysts. $TLR4^{-/-}$ mice were more susceptible to infection compared with $TLR2^{-/-}$ and C57BL/6 mice. Nuclear factor-kappa B activation through TLR4 agonistic activity of *T. gondii* ME49 was demonstrated by luciferase assay using stably expressing mouse (m) TLR2 or mTLR4/mMD-2 transfectants. We demonstrate here for the first time that innate immune recognition by TLR4 is involved in protective mechanisms against peroral infection with *T. gondii* ME49. These results suggest that the small intestine plays an important role in the induction of innate immunity in naturally acquired toxoplasmosis.

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite occurring worldwide in both human and animal hosts. Under normal conditions, infection is largely asymptomatic. However, in immunocompromised hosts, such as patients with AIDS, the parasite can become widely disseminated and cause severe toxoplasmosis or encephalitis (1). Natural infection with *T. gondii* is acquired orally by ingestion of undercooked or raw meat-containing parasite cysts or oocysts. Following ingestion, bradyzoites or sporozoites are released from the cysts and rapidly invade the intestinal mucosa and convert into tachyzoites. After multiplication and disruption of infected cells, tachyzoites invade neighboring cells and disseminate via the blood and lymphatic system. For reasons mentioned above, the peroral route of *T. gondii* infection is thought to reflect the typical infection that occurs in naturally acquired toxoplasmosis in humans, and it is important to investigate the mechanism of host resistance against the parasite using animals infected via peroral route.

Recently, it has been reported that toll-like receptor 2 (TLR2) and the TLR4/MD-2 complex are highly expressed in the gastrointestinal mucosa of mice, and play important roles in innate immunity against pathogens (2, 3). The TLR family

Correspondence to: T. Furuta; E-mail: furuta@ims.u-tokyo.ac.jp

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has been identified as key host molecules in the induction of innate immune responses to microbial ligands (4, 5). TLR2 and TLR4 react to bacterial cell-wall compounds (6, 7). TLR2 is activated by a variety of ligands, such as bacterial lipopeptides, as well as fungal and mycobacterial components (8), while TLR4 is activated not only by bacterial LPS but also apparently by other ligands, such as viral proteins (9–11). So far, little information is available regarding roles of the small intestine in the induction of innate immunity mediated by TLRs in naturally acquired toxoplasmosis.

In the present study, we therefore decided to investigate the possible differential role of TLR2 and TLR4 in mediating host defense against *T. gondii* infection, using animal models of peroral infection with *T. gondii*. Our results indicate that TLR4 mediates innate immunity against toxoplasmosis induced by peroral, but not intra-peritoneal infection of *T. gondii* ME49 cysts, and that the small intestine plays an important role in this process.

Methods

Experimental animals

$TLR2^{-/-}$ and $TLR4^{-/-}$ mice with C57BL/6 background (12, 13) were bred and maintained under SPF conditions at the Animal Center, Institute of Medical Science, University of Tokyo, Japan. C57BL/6, TLR4-deficient C3H/HeJ and the wild-type (WT) C3H/HeN mice were purchased from Japan SLC (Hamamatsu, Japan). Female mice between 5 and 6 weeks old were used for experiments. The animal experiments were approved by the Committee for Animal Experiment of the Institute of Medical Science, University of Tokyo.

Parasites and experimental infection

Brain tissue containing *T. gondii* ME49 cysts was prepared by homogenization in saline, and the homogenate was used as an inoculum for infection after the number of cysts in homogenates had been counted. Mice were infected with various doses of cysts of the avirulent *T. gondii* ME49 strain, by peroral or intra-peritoneal inoculation (p.o.i. or i.p.i.). The number of cysts in infected brains was determined by microscopic examination of brain homogenates made with saline. Brains from uninfected control mice were also removed in parallel, and an equal volume of infected brains was administered by p.o.i. or i.p.i. as a control. *Toxoplasma gondii* ME49 tachyzoites were obtained by tissue culture using Vero cells as previously described, and they were used as a soluble *Toxoplasma gondii* ME49 tachyzoite antigen (SA) after solubilization by sonication (14). To examine the possibility that normal Vero cell lysate induces cytokine production or nuclear factor-kappa B (NF-κB) luciferase activity, cytokine production or NF-κB luciferase activity was also performed using normal Vero cell lysate. Since their activities were comparable with those of controls (medium alone), it was concluded that contamination of normal Vero cells in SA did not significantly affect cytokine production or NF-κB luciferase activity.

Measurement of cytokine production in spleen cells

For measurement of *in vitro* cytokine production, single-cell suspensions were prepared from spleens of *T. gondii* ME49-infected mice at 5 days after p.o.i. or i.p.i. Spleen cells were cultured at 1×10^6 cells per well in 1 ml RPMI1640 containing 10% FCS and antibiotics with or without SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. The levels of IFN-γ, IL-4, IL-6, IL-10 and IL-12p40 in the culture supernatants were measured by sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Samples were assayed in triplicate, and the data were expressed as the mean ± SD.

Histopathology and immunohistochemistry

The major organs and small intestines of mice were fixed in 10% neutral buffered formalin and then embedded in paraffin. Sections were made and stained with H&E for evaluation of pathologic changes. For immunohistochemistry, deparaffinized sections were stained with anti-mouse TLR2- or TLR4/MD-2-specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were then washed in PBS and incubated with anti-rat antibody labeled with biotin, streptavidin labeled with peroxidase (Histofine, Nichirei Corp., Tokyo, Japan) and then incubated with diaminobenzidine substrate. Normal rabbit IgG instead of primary antibody was used as a negative control. Finally, sections were mounted in Mount-Quick (Daido Sangyo, Co., Ltd, Tokyo, Japan) and then observed. Quantification of mononuclear cell or neutrophil numbers in the lesions of small intestine was determined by counting these cells in 20 high-magnification fields of sections, and the results were presented as fold increase as compared with normal mice.

Luciferase assay

The Ba-F3 cell line stably expressed mouse (m) TLR2 or mTLR4/mMD-2 and p55Ig κLuc with an NF-κB-dependent luciferase reporter construct (15). For the luciferase assay, the transfectants were washed and cultured in RPMI1640 containing 10% FCS and IL-3. Individual wells containing 1×10^5 cells were left untreated or stimulated with SA. In some experiments, SA was pre-treated with 10 mg of polymyxin B (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 37°C for 2 h to deactivate LPS activity. LPS (100 ng ml^{-1}, Sigma, St Louis, MO, USA) or Pam_3CSK_4 (100 ng ml^{-1}, ECM PG Microcollections) was used as a ligand for TLR4 or TLR2. After 4 h stimulation with ligand or SA, cells were harvested, washed and then lysed in 100 μl lysis buffer. Luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Nippon Gene, Toyama, Japan), quantified as relative light units on a luminometer (Berthold Japan, Tokyo, Japan). The results were presented as fold increase as compared with control wells.

Antibiotics

Penicillin G potassium salt (1500 U ml^{-1}, Wako Pure Chemical Industries, Ltd) and streptomycin sulfate (2 mg ml^{-1}, Wako Pure Chemical Industries, Ltd) were administered via sterilized drinking water to remove the intestinal microflora of mice until fecal bacteria disappeared. During the treatment with antibiotics, cages, bottles for drinking water and antibiotic solutions given to the mice were renewed daily. Feces from each cage of mice were collected individually and placed

into sterile tubes. The fecal bacteria were checked daily by Giemsa stain to examine the effect of antibiotic treatment. Fecal bacteria had disappeared at 3 days after the treatment, and then *Toxoplasma* infection was performed.

Statistical analyses

Statistical significance was established using an unpaired, two-tailed Student's *t* test. Statistically significant data are indicated in the figures by an asterisk, and the corresponding *P* values are listed in the figure legends.

Results

Number of brain cysts in C3H/HeJ and C3H/HeN mice infected with T. gondii ME49

Since C3H/HeJ mice are functionally TLR4-deficient for LPS signaling, we sought to determine whether these mice were susceptible to *Toxoplasma* infection compared with WT C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with *T. gondii* ME49, either by p.o.i. as a natural route of infection or by i.p.i. as an unnatural route of infection. The number of brain cysts was examined 30 days after infection. The number of brain cysts in C3H/HeJ mice infected by p.o.i was significantly higher than C3H/HeN mice, suggesting that TLR4 might be required for innate resistance to murine toxoplasmosis. In striking contrast, in both strains following i.p.i. of *T. gondii* cysts, the number of cysts in the brains was low and not significantly different between the two mouse strains (Fig. 1). This indicates that TLR4-mediated innate resistance was not involved in the response to i.p.i. of *T. gondii*. Since innate immunity mediated by TLR4 might contribute to host resistance to *T. gondii* infection when parasites are inoculated via the peroral route but not the intra-peritoneal route, it appears that induction of innate resistance to murine toxoplasmosis is dependent on the route of parasite inoculation.

Production of pro-inflammatory cytokines in C3H/HeJ and C3H/HeN mice infected with T. gondii ME49

To compare the level of pro-inflammatory cytokines, IFN-γ, IL-12 and IL-6 produced in response to *T. gondii* infection,

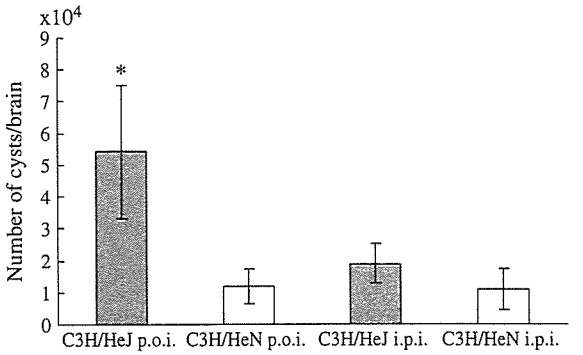

Fig. 1. Growth of *T. gondii* ME49 cysts in the brains of TLR4-deficient C3H/HeJ and WT C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with 10 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then the number of cysts in the brain was examined at 30 days after the infection (mean ± SD, $n = 6$, $^*P < 0.01$). This experiment was repeated three times with similar results.

spleen cell cultures were prepared at 5 days after infection and cultured with SA for 24 h. Supernatants were collected from each well, and then cytokine production was measured by sandwich ELISA. When C3H/HeJ and C3H/HeN mice were inoculated perorally with *T. gondii* ME49 cysts, the production of IFN-γ and IL-12 in C3H/HeJ (TLR4-deficient) mice was significantly lower than that in C3H/HeN mice. However, when these mice were inoculated intra-peritoneally with *T. gondii* ME49 cysts, IFN-γ and IL-12 were produced at the same level in both strains, and their levels were almost comparable with those of C3H/HeN mice with p.o.i. of *T. gondii* ME49, which showed resistance to *T. gondii* infection (Fig. 2). These results suggest that increased susceptibility to *T. gondii* in C3H/HeJ mice infected perorally is associated with reduced levels of IFN-γ and IL-12, which are the key factors for resistance to murine toxoplasmosis. Regarding IL-6 production, the level of IL-6 was significantly lower in C3H/HeJ (TLR4-deficient) mice than that in C3H/HeN mice after p.o.i. of cysts; however, IL-6 production was low in both C3H/HeJ and C3H/HeN mice following i.p.i. of *T. gondii*.

Histopathology and immunohistochemistry

In histopathologic examinations following peroral infection with *T. gondii* in C3H/HeJ and C3H/HeN mice, both necrosis and remarkable cellular inflammation were observed in the small intestine of C3H/HeJ (TLR4-deficient) mice at 5 days post-infection. Although the infiltration of neutrophils in the small intestines of C3H/HeJ and C3H/HeN mice was comparable, the infiltration of monocytes in C3H/HeJ mice was less prominent than that in C3H/HeN mice (Fig. 3A and B). However, such inflammatory changes were usually not marked in either C3H/HeJ or C3H/HeN mice following i.p.i. of *T. gondii* ME49. After p.o.i. of cysts, TLR4 was expressed in mononuclear cells in the lamina propria of the intestines. The number of TLR4-positive cells was increased in resistant C3H/HeN mice (Fig. 3C, arrows) compared with susceptible C3H/HeJ mice (Fig. 3D). It was hypothesized that the appearance of TLR4-positive cells might have been induced by the infection in C3H/HeN mice and reflects innate immunity to *T. gondii*. However, when C3H/HeJ or C3H/HeN mice were infected by i.p.i. of parasites, TLR4-positive cells were not usually observed in the lamina propria (Fig. 3E and F). TLR2-positive cells were not observed in C3H/HeJ and C3H/HeN mice after p.o.i. of cysts (Fig. 3G and H). No TLR2 or TLR4 immunoreactivity was found in the small intestine of normal C3H/HeJ and C3H/HeN mice (data not shown). The small intestines of *T. gondii*-infected C3H/HeJ and C3H/HeN mice were also not stained when control rabbit IgG was used as the primary antibody (data not shown). To quantify inflammatory cell recruitment, the number of mononuclear cells or neutrophils in the small intestinal lesions was examined by counting these cells in minimum of 20 high-magnification fields of sections, and the results were expressed as fold increase compared with those of normal C3H/HeJ and C3H/HeN mice. Following p.o.i. of *T. gondii* ME49, the number of mononuclear cells infiltrated in C3H/HeJ (TLR4-deficient) mice was significantly lower than that in C3H/HeN mice, although the infiltration of neutrophils in the lesion was similar in C3H/HeJ and C3H/HeN mice (Fig. 4).

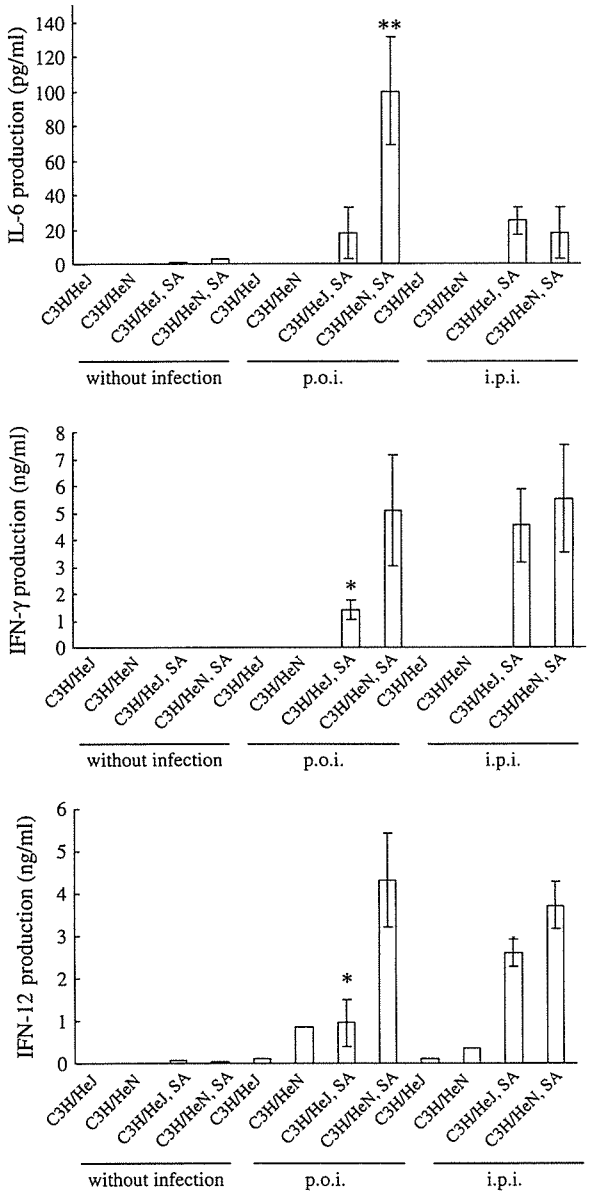

Fig. 2. Production of IFN-γ, IL-12 and IL-6 in spleen cells from *T. gondii* ME49-infected C3H/HeJ and C3H/HeN mice. C3H/HeJ and C3H/HeN mice were infected with 10 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then single-cell suspensions were prepared from spleens of the mice at 5 days after the infection. Spleen cells were cultured at 1×10^6 cells per well in 1 ml RPMI1640 containing 10% FCS in the presence or absence of SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. The concentration of IFN-γ, IL-12 and IL-6 in spleen cells was measured by sandwich ELISA (mean ± SD, $n = 5$, $*P < 0.05$, $**P < 0.01$). This experiment was repeated three times with similar results.

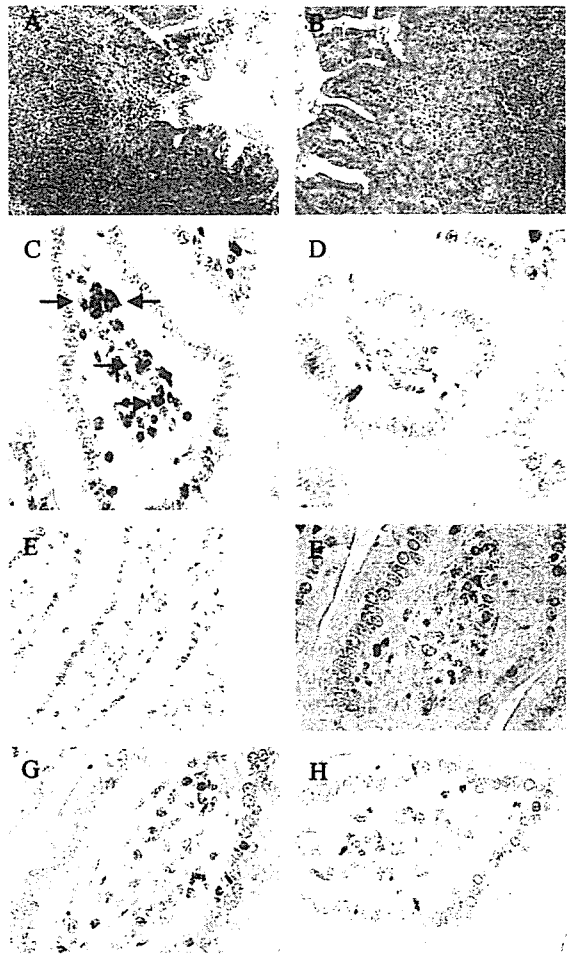

Fig. 3. Histopathology of the small intestines in C3H/HeJ and C3H/HeN mice infected with *T. gondii* ME49. In histopathologic examinations following peroral infection with *T. gondii* in C3H/HeJ and C3H/HeN mice, necrosis and remarkable cellular inflammation were observed in the small intestine of C3H/HeJ (TLR4-deficient) mice at 5 days post-infection (A and B, original magnification ×200). Although the infiltration of neutrophils in the small intestines of C3H/HeJ and C3H/HeN mice was comparable, the infiltration of monocytes in C3H/HeJ mice was less prominent than that in C3H/HeN mice. However, such inflammatory changes were usually not marked in either C3H/HeJ or C3H/HeN mice following i.p.i. of *T. gondii* ME49. TLR4-positive cells stained by anti-TLR4 antibody were observed in the lamina propria of C3H/HeN mice (C, arrows, original magnification ×400), but these cells were few or absent in the lamina propria of C3H/HeJ mice (D, original magnification ×400). No positive cells stained by anti-TLR4 antibody were observed in the lamina propria of C3H/HeJ and C3H/HeN mice infected by i.p.i. with *T. gondii* ME49 cysts (E and F, original magnification ×400). No positive cells stained by anti-TLR2 were observed in the small intestines of C3H/HeJ and C3H/HeN mice with p.o.i. of cysts (G and H, original magnification ×400). No TLR2 or TLR4 immunoreactivity was found in the small intestine of normal C3H/HeJ and C3H/HeN mice (data not shown). The small intestines of *T. gondii*-infected C3H/HeJ and C3H/HeN mice were also not stained when control rabbit IgG was used as the primary antibody (data not shown).

Differential roles of TLR2 and TLR4 in mediating the induction of host defense to T. gondii infection

To confirm further the possible differential role of TLR2 and TLR4 in mediating the induction of innate immunity against *T. gondii* infection, $TLR2^{-/-}$ or $TLR4^{-/-}$ mice were inoculated by p.o.i. or i.p.i. with 20 cysts of *T. gondii* ME49 to examine the growth of brain cysts in animals. When cysts were inoculated by p.o.i., a significant increase of parasites was found in the

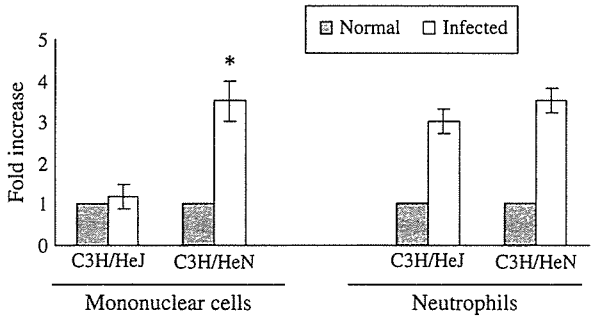

Fig. 4. Number of mononuclear cells and neutrophils in the lamina propria of the small intestine. Number of mononuclear cells and neutrophils in the small intestine of C3H/HeJ and C3H/HeN mice was counted by microscopy in 20 high-magnification fields of sections stained with H&E at 5 days after peroral *T. gondii* ME49 infection. Data were presented as fold increase as compared with the infected C3H/HeJ mice (mean ± SD, $n = 5$, $^*P < 0.01$).

TLR4$^{-/-}$ mice compared with TLR2$^{-/-}$ or WT C57BL/6 mice at 30 days after the infection (Fig. 5A). However, when cysts were inoculated by i.p.i., the number of brain cysts in TLR4$^{-/-}$ mice was comparable to those of TLR2$^{-/-}$ and WT mice. To further evaluate the role of TLR2 and TLR4 in host resistance to *T. gondii*, TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected with different doses of *T. gondii* ME49 cysts, by p.o.i. or i.p.i. as indicated in Fig. 5(B and C). When 20 cysts were inoculated by p.o.i., TLR4$^{-/-}$ mice were more susceptible than TLR2$^{-/-}$ or WT mice. All TLR4$^{-/-}$ mice died between 34 and 38 days after parasite inoculation, whereas TLR2$^{-/-}$ or WT mice survived >45 days (Fig. 5B). However, these differences in susceptibility between TLR2$^{-/-}$ and TLR4$^{-/-}$ mice disappeared when mice were inoculated with a higher dose of cysts (200 cysts); all mice died within 15 days after infection (Fig. 5B). In contrast to p.o.i., no remarkable differences were observed between TLR2$^{-/-}$ and TLR4$^{-/-}$ mice receiving low- or high-dose i.p.i. (40 or 200 cysts, Fig. 5C). When TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were inoculated with low dose of cysts by i.p.i., all mice survived >45 days, while those mice inoculated with high dose of cysts by i.p.i., all died within 15 days. These data suggest that TLR4 serves as a protective function in the host response to *Toxoplasma* infection, and that TLR4 signaling is required to control the growth of *T. gondii* infection when parasites are inoculated by p.o.i.

Production of pro-inflammatory cytokines in TLR4$^{-/-}$ mice infected by p.o.i. of T. gondii ME49

To compare the level of cytokines (IFN-γ, IL-4, IL-6, IL-10 and IL-12) produced in response to *T. gondii* infection, spleen cells were prepared at 5 days after the infection of *T. gondii* ME49 cysts by p.o.i., and cultured with SA for 24 h. Supernatants were collected, and then cytokine production was measured by sandwich ELISA. When TLR4$^{-/-}$ mice were inoculated by p.o.i. with *T. gondii* ME49 cysts, the production of IFN-γ, IL-12 and IL-6 in TLR4$^{-/-}$ mice was significantly lower than that in TLR2 or WT mice, which are resistant to murine toxoplasmosis, although production of IL-4 and IL-10 in TLR4$^{-/-}$ was comparable with those in TLR2$^{-/-}$ and WT mice. These results suggest that the increased production of

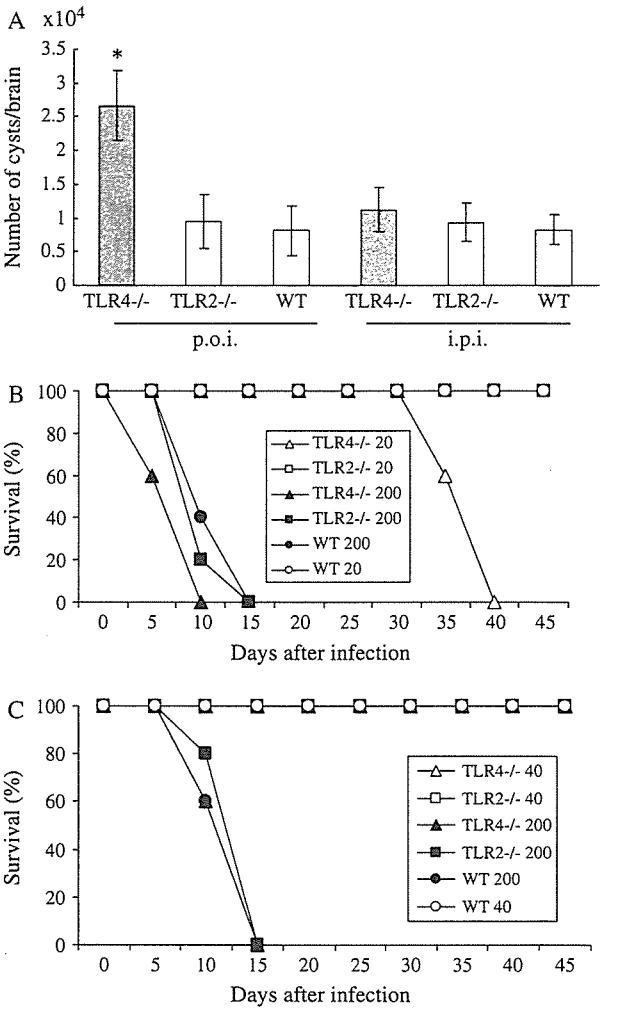

Fig. 5. Growth of *T. gondii* ME49 cysts in the brains of TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice infected by p.o.i. or i.p.i. (A) TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected with 20 cysts of *T. gondii* ME49 by p.o.i. or i.p.i., and then number of cysts in the brain was examined at 30 days after the infection (mean ± SD, $n = 5$, $^*P < 0.05$). This experiment was repeated three times with similar results. Survivals of TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice after *T. gondii* ME49 infection. TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected perorally (B) or intra-peritoneally (C) with 20 cysts of *T. gondii* ME49 as indicated in each figure, and then the survival was monitored. Data are representative of three times experiments, each with five female mice per group.

IFN-γ, IL-12 and IL-6 mediated by TLR4 is associated with resistance to murine toxoplasmosis (Fig. 6) and is consistent with the data showing that production of cytokines in C3H/HeJ (TLR4-deficient) mice infected perorally with *T. gondii* is lower than that in C3H/HeN mice (Fig. 2).

Induction of TLR4-dependent signaling pathway by T. gondii ME49 infection

To further characterize the activation of TLR4 by *T. gondii* ME49, a stable transfectant-expressing mTLR2 or mTLR4/mMD-2 was stimulated with SA to examine whether *T. gondii*

ME49 influences signaling via mTLR4/mMD-2, and then NF-κB activation was examined by luciferase assay. SA induced NF-κB activation in a dose-dependent manner in the transfectant-expressing mTLR4/mMD-2 but not in the transfectant-expressing mTLR2 (Fig. 7). These activities of SA were not abolished by treatment with polymyxin B to deactivate LPS. These results suggest that *T. gondii* ME49 has a TLR4 agonistic activity.

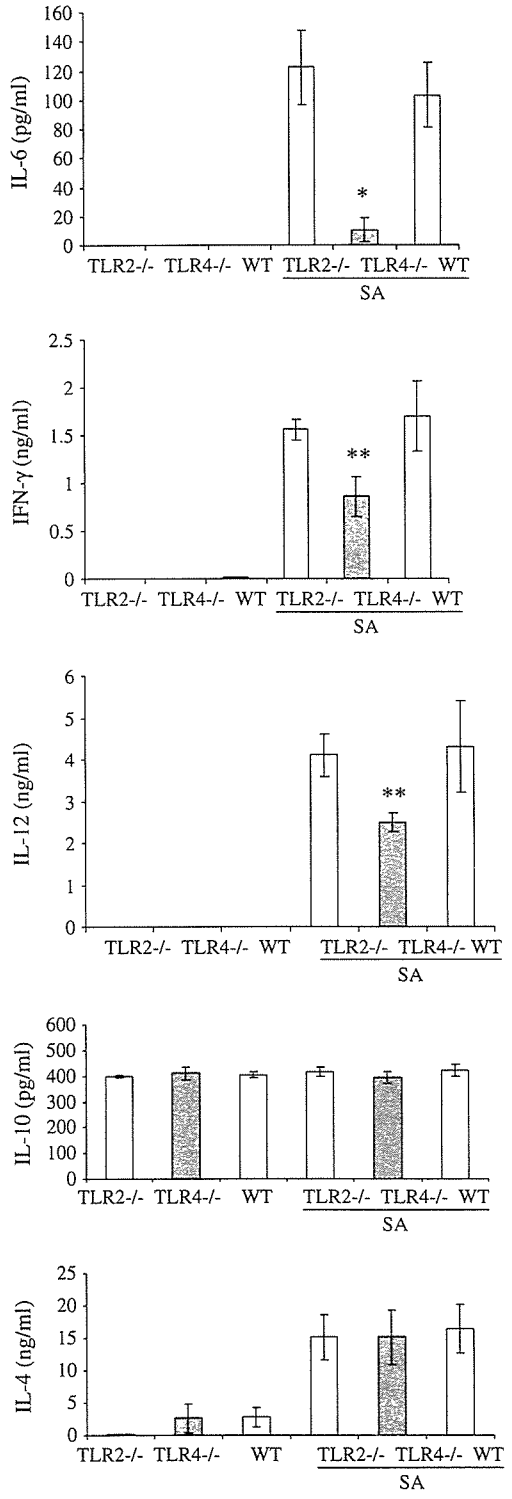

Fig. 6. Production of IFN-γ, IL-4, IL-6, IL-10 and IL-12 in spleen cells from TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice after p.o.i. of *T. gondii* ME49. TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice were infected orally with 20 cysts of *T. gondii* ME49, and then single-cell suspensions were prepared from spleens of the mice at 5 days after the infection. Spleen cells were cultured at 1 × 10^6 cells per well with or without SA (20 μg ml^{-1}), and then supernatants were collected 24 h later. Concentration of IFN-γ, IL-4, IL-6, IL-10 and IL-12 in spleen cells from the infected mice was measured by sandwich ELISA (mean ± SD, $n = 5$, $^*P < 0.01$, $^{**}P < 0.05$). The cytokine production in spleen cells from normal TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice was also examined by sandwich ELISA as mentioned before, but the production of cytokines was not detected in these normal mice (data not shown). This experiment was repeated three times with similar results.

Influence of normal intestinal microflora on TLR4-mediated innate resistance against T. gondii ME49 infection

To consider the possibility that the normal intestinal microflora, which is mostly gram-negative bacteria, affects TLR4-mediated innate resistance during peroral infection with *T. gondii*, penicillin G and streptomycin sulfate were administered via sterilized drinking water to remove intestinal microflora of mice. After TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice had been treated with antibiotics, *T. gondii* cysts were inoculated orally into these mice, and then the number of brain cysts was examined. Since a high susceptibility to *T. gondii* infection was still observed in TLR4$^{-/-}$ mice treated with antibiotics compared with TLR2$^{-/-}$ or WT mice (Fig. 8), we conclude that TLR4-mediated host

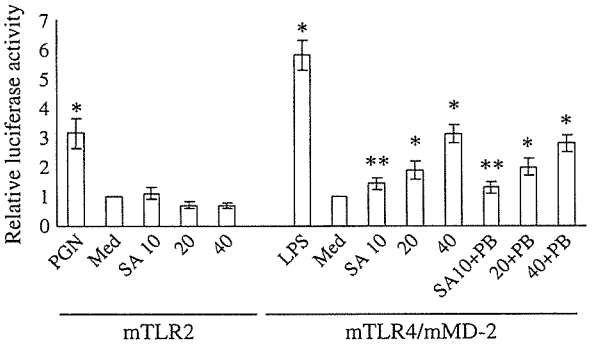

Fig. 7. Induction of TLR4-dependent signaling pathway by *T. gondii* ME49. Ba-F3-transfected cells were cultured in RPMI1640 containing 10% FCS and IL-3. The Ba-F3 cell line was stably expressing mouse (m) TLR2 or mTLR4/mMD-2 and p55Ig κLuc with NF-κB-dependent luciferase reporter construct. Individual wells containing 1 × 10^5 cells were left untreated or stimulated with SA (20 μg ml^{-1}) as indicated. Luciferase assays were performed using the Luciferase Assay System according to the manufacturer's instructions. LPS (100 ng ml^{-1}) or Pam_3CSK_4 (PCSK, 100 ng ml^{-1}) was added as positive control for mTLR4 or mTLR2. In some experiments, SA was pretreated with 10 mg of polymyxin B (PB) at 37°C for 2 h to deactivate LPS activity (mean ± SD, $^*P < 0.01$ and $^{**}P < 0.05$ for mTLR2 or mTLR4/mMD-2 without SA). The results were presented as fold increase as compared with control wells. The experiment was performed three times with similar results.

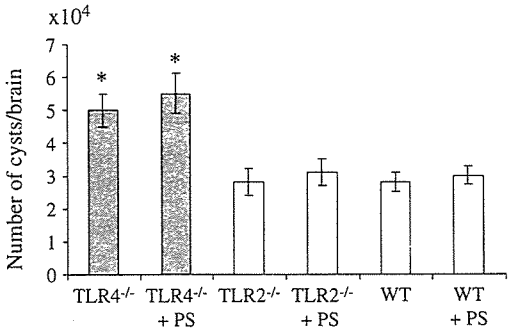

Fig. 8. Influence of normal intestinal microflora on TLR4-mediated innate resistance against *T. gondii* ME49 infection. After $TLR2^{-/-}$, $TLR4^{-/-}$ and WT mice were treated with penicillin G (1500 U ml^{-1}) and streptomycin sulfate (2 mg ml^{-1}) (PS) via sterilized drinking water, 20 cysts of *T. gondii* were inoculated orally into these mice, and then the number of brain cysts was examined at 30 days after the infection (mean ± SD, $n = 5$, $^*P < 0.01$ for $TLR2^{-/-}$or WT mice with or without antibiotics). Data are representative of three times experiments.

resistance is not influenced by the presence of normal intestinal microflora.

Discussion

We demonstrate here that innate immune recognition by TLR4 is involved in protective mechanisms against naturally acquired *T. gondii* ME49 infection via the oral route. In addition, the small intestine plays important roles in this process. Since the peroral route of *T. gondii* infection has been considered to reflect the typical infection route that occurs in naturally acquired toxoplasmosis in humans, it is important to study the mechanism of resistance against the parasite using animals infected via peroral route. We therefore decided to characterize the potential role of innate immunity against toxoplasmosis in mice infected by p.o.i. of *T. gondii* ME49.

To investigate the role of the TLR in host defense against *T. gondii*, we infected both TLR-deficient and WT mice with *T. gondii* ME49. Upon i.p.i., TLR4-deficient C3H/HeJ and control C3H/HeN mice had almost identical number of cysts on the brain and produced similarly high amounts of IFN-γ and IL-12, which are the key factors for resistance to *T. gondii* infection. This suggests that innate immunity mediated by TLR4 was not critical in these mice. In striking contrast, upon p.o.i. with *T. gondii* ME49, C3H/HeJ mice were significantly more susceptible to *T. gondii* infection than C3H/HeN mice. This was associated with a significant decrease of pro-inflammatory response in C3H/HeJ mice as evidenced by reduced production of IFN-γ and IL-12 at 5 days after infection. These results might suggest that IFN-γ and IL-12 production in mice after peroral infection is mediated by TLR4 and confers resistance to the infection. In the case of i.p.i. of parasites, it is likely that immunity is assessed in central lymphoid organs (16). Since intra-peritoneal infection with *T. gondii* leads to rapid induction of a strong systemic immune response (such as cell-mediated immunity), it is possible that the local immune response (such as innate immunity in the small intestine) is limited following intra-peritoneal infection of mice (17, 18). Regarding mucosal immune responses of the small intestine, we have so far examined IFN-γ production in lamina propria cells as well as spleen cells after p.o.i. of *T. gondii* ME49 cysts. Although increased IFN-γ production by lamina propria cells in C3H/HeN mice was consistently observed as compared with C3H/HeJ mice (data not shown), these differences were not significant. The reasons why significant differences were not observed between C3H/HeJ and C3H/HeN mice were not clear. Despite our careful attention, we cannot rule out the possibility that, during the preparation of lamina propria cells from the small intestines, there may have been contamination with small amounts of bacteria or bacterial components such as LPS or other toxins that then influenced cytokine production.

To confirm further the importance of TLR4 for resistance to *T. gondii* infection, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were inoculated perorally or intra-peritoneally with *T. gondii* cysts. $TLR4^{-/-}$ but not $TLR2^{-/-}$ mice proved to be susceptible to *T. gondii* infection when parasites were inoculated by peroral route (Fig. 5). These data clearly implied that TLR4 is involved in host resistance against murine toxoplasmosis via the peroral route of infection.

Recently, other researchers have reported the importance of innate immunity against *Toxoplasma* parasites using murine models. Scanga *et al.* (19) reported that the induction of IL-12 by *T. gondii* depends on a unique mechanism involving both MyD88 and G protein-coupled signaling pathways, without participation of TLR2 or TLR4 signaling. On the other hand, Mun *et al.* (20) reported that the effect of TLR2 on survival of *T. gondii* Fukaya-infected mice is dependent on the dose of *T. gondii*: TLR2 is not an essential molecule for protective immunity to low-dose infections (50 and 100 cysts), but is essential for protective immunity to high-dose infections of *T. gondii* (≥300 cysts). In the present study, we inoculated 20 cysts of *T. gondii* ME49 into mice via the intra-peritoneal route, and found that the number of brain cysts in $TLR2^{-/-}$ mice was comparable with $TLR4^{-/-}$ and WT mice (Fig. 5A). For a low-dose infection, this suggests that protective immunity to *T. gondii* ME49 is not dependent on TLR2, which may be consistent with the data reported by Mun *et al.* (20). However, there were several differences in the experimental conditions between the studies of Mun *et al.* and our own. They inoculated intra-peritoneally with cysts of *T. gondii* Fukaya stain, and the number of brain cysts in mice was estimated using PCR amplification of the SAG1 gene at 8 days after the infection. Cytokine production *in vitro* was measured in peritoneal macrophages stimulated with live bradyzoites of *T. gondii* Fukaya at 1 day after the infection. On the other hand, we used cysts of *T. gondii* ME49 strain, and the number of cysts in brain homogenates was counted directly by microscopy at 30 days after infection. We measured cytokine productions in the spleen cells with stimulation of sonicated SA at 5 days after the infection. The differences of experimental conditions might correlate with the discrepancies of data reported.

In addition to these innate resistances to *T. gondii* infection, recent data reported by Yarovinsky *et al.* (21) indicate that *T. gondii* profilin activates dendritic cells through TLR11 and that TLR11 is required *in vivo* for parasites-induced IL-12 production and optimal resistance to infection. Furthermore, Debierre-Grockiego *et al.* (22) reported that glycosylphosphatidylinositol molecules in *T. gondii* stimulate cytokine

production in macrophages, and they can serve as TLR2 as well as TLR4 agonists (23).

In the present study, remarkable histopathological changes in the small intestine were found in mice infected with *T. gondii* ME49 by p.o.i. but not i.p.i. These findings are consistent with data previously reported, showing that local immune responses in the gut during acute phase infection are limited following intra-peritoneal infection (18, 24). *Toxoplasma gondii*-infected TLR4-deficient C3H/HeJ mice showed an increasing infiltration of neutrophils with necrosis and low number of mononuclear cells (including TLR4-positive cells) in the small intestine, together with increased numbers of brain cysts (Fig. 4). This finding might be correlated with data suggesting macrophage defects and dysfunction of inflammatory cell recruitment in C3H/HeJ mice susceptible to *Salmonella* infection (25). Severe neutrophil infiltration in the small intestine of C3H/HeJ mice was found after p.o.i., suggesting TLR4-independent neutrophil migration. Haziot *et al.* (26) demonstrated that dramatically enhanced neutrophil recruitment in response to either LPS or *Escherichia coli* is TLR4 independent. It is not clear why the number of TLR4-expressing mononuclear cells was increased during the infection. However, Ortega-Cava *et al.* (2) reported that the TLR4/MD-2 complex is mainly found in mononuclear cells that infiltrated two different areas of the mucosa, crypt epithelial cells and lamina propria, and that TLRs and protein levels, as well as CD14, are up-regulated during dextran sodium sulfate-induced inflammation. After oral infection with cysts, bradyzoites are released from cysts and rapidly invade the intestinal epithelial and lamina propria cells (27). In the present study, TLR4-positive cells increased in the lamina propria after p.o.i. and were associated with resistance to infection as shown by decreased numbers of cysts and high cytokine production in C3H/HeN mice. We postulate that the increase of TLR4-expressing cells in the small intestine might reflect resistance to *T. gondii* infection generated by the mucosal immune system.

Acknowledgements

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Abbreviations

i.p.i.	intra-peritoneal inoculation
NF-κB	nuclear factor-kappa B
p.o.i.	peroral inoculation
SA	soluble *Toxoplasma gondii* ME49 tachyzoite antigen
TLR	toll-like receptor
WT	wild-type

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REVIEW *Public Health*

Bovine Spongiform Encephalopathy (BSE) Safety Measures in Japan

Kazuya YAMANOUCHI[1] and Yasuhiro YOSHIKAWA[2]

[1]Nippon Institute for Biological Science, 9–2221–1 Shin-machi, Ome, Tokyo 198–0024 and [2]Department of Biomedical Science, Graduate School of Agriculture and Life Science, The University of Tokyo, Tokyo 113–08657, Japan

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ABSTRACT. Since the first identification of bovine spongiform encephalopathy (BSE) in Japan in September 2001, a series of safety measures was introduced by the Ministry of Agriculture, Forestry and Fisheries, the Ministry of Health, Labour and Welfare and the Food Safety Commission of the Cabinet Office. These measures included blanket BSE testing and removal of specified risk materials at slaughterhouses, surveillance of risk animals and a ban on the use of meat-and-bone meals and traceability on all farms. The Japanese experience over the past five years has shed light on several issues in countries that have a low BSE incidence.
KEY WORDS: bovine spongiform encephalopathy, control, Japan, public health, risk assessment.

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The first case of bovine spongiform encephalopathy (BSE) in Japan was identified in September 2001. In consequence, a series of safety measures were introduced and implemented by three government bodies, as follows:

— the Ministry of Agriculture, Forestry and Fisheries (MAFF), responsible for disease control of cattle on farms
— the Ministry of Health, Labour and Welfare (MHLW), responsible for cattle in slaughterhouses
— the Food Safety Commission of the Cabinet Office, created in July 2003, and its Prion Expert Committee, responsible for the assessment of BSE-related risk.

The measures which were enforced to assess and manage BSE risk by these authorities are summarised in Table 1.

As of May 2006, a total of 9 cases were diagnosed in Japan by active surveillance of cattle on farms and 18 cases through screening tests of all cattle in slaughterhouses.

As in any other country, the occurrence of the first BSE case had a nationwide effect on consumer confidence towards the safety of beef. However, the measures implemented by the government after the diagnosis of the first BSE case, especially after blanket BSE testing of all cattle in slaughterhouses since October 2001, have been successful in restoring confidence. Japanese experience in the control of BSE over the past five years has shed light on several issues in countries that have a low BSE incidence rate.

HISTORICAL SUMMARY OF PRION DISEASES IN JAPAN

In 1976, research on prion diseases in Japan commenced with the establishment of the Slow Virus Research Committee by the MHLW. As the first mouse model of Creutzfeldt-Jakob disease (CJD) was developed at that time [11], the Committee mainly focused on CJD. In the 1980s, scrapie became another focus of interest after the isolation of the scrapie agent from sheep imported from Canada [9]. The results of the work performed by this research group contributed to the scientific basis for subsequent BSE measures, such as the development of immunohistochemistry techniques in mid-1980s [6]. The Committee is still active and now places special emphasis on CJD.

In 1997, in response to the publication of the first cases of variant Creutzfeldt-Jakob (vCJD) disease in the United Kingdom, the MAFF and MHLW formed independent research groups to investigate prion diseases, with members of the above-mentioned research committee as core researchers. The studies of these groups have covered both basic and practical aspects of prion diseases, such as pathogenesis and diagnosis, as well as decontamination. The Western blot technique was improved and a panel of monoclonal antibodies against prion protein was produced as a result of their research [4, 10]. Thus, these two research groups contributed significantly to the implementation of safety measures in Japan when the first BSE case was identified. Both groups are still active.

Passive surveillance of BSE commenced in April 1996. In April 2001, active surveillance of farm cattle showing neurological signs was initiated using rapid commercial BSE test kits. As only a small number of samples (less than 300) had been obtained by the end of April 2001, the criteria for sample collection were expanded and all animals that were recumbent were included in the target population. During surveillance, a five-year-old dairy cow was found to be recumbent at a slaughterhouse and was confirmed as a BSE case on 10 September 2001 [5, 13].

On 27 September 2001, a series of food safety measures, including the removal of specified risk materials (SRM), was introduced. On 4 October, a complete ban on the use of meat-and-bone meal (MBM) was implemented by MAFF.

On 2 October 2001, a training course on the Bio-Rad antemortem BSE test kit (an ELISA test) was held for meat inspectors at 117 meat inspection centres under the supervision of the MHLW. Upon completion of the ELISA training course, the screening programme for BSE was introduced at slaughterhouses for cattle of all ages on 18 October 2001.

Table 1. Risk assessment and management for bovine spongiform encephalopathy in Japan

Date		Ministry of Agriculture, Forestry and Fisheries	Ministry of Health, Labour and Welfare	Food Safety Commission
1996	April	Passive surveillance Administrative guidance on feed ban of MBM for cattle		
1997	April	BSE designated a notifiable disease in the contagious diseases of domestic animals prevention law		
2001	Apr	Active surveillance		
	Oct	Complete ban of importation of MBM Complete feed ban of MBM for all animals Enhanced active surveillance	Removal of SRM (CNS, distal ileum, eye) BSE testing on cattle of all ages in slaughterhouses	
2003	July			Creation of Food Safety Commission
	Dec	Bovine traceability system at production level		
2004	Feb		Vertebral column included in SRM	
	Apr	BSE test for all dead cattle over 24 months mandatory		
	Sept			Comprehensive review of measures on BSE in Japan
	Dec	Bovine traceability system at market distribution level		
2005	Mar	All feed factories have exclusive production lines		
	May			Food safety risk assessment related to BSE measures (change of testing age)
	Aug	Enforcement of notification of imported compound feed	Age of BSE testing changed (cattle over 21 months)	
	Dec			Risk assessment of imported beef from USA and Canada

MBM meat-and-bone meal.
SRM specified risk materials.
CNS central nervous system.
BSE bovine spongiform encephalopathy.

NUMBER OF BSE CASES IN JAPAN

Between October 2001 and the end of March 2006, a total of 5,527,913 cows were screened in slaughterhouses. By 16 May 2006, a total of 18 BSE cases had been confirmed (http://www.mhlw.go.jp/houdou/0110/h1018-6.html). Surveillance of animals at risk first covered limited numbers but reached 100% by April 2004. In 2005, a total of 95,310 head of cattle were tested, and 3 BSE cases were confirmed. By the end of March 2006, surveillance had covered the testing of a total of 247,781 cows. As of May 2006, a total 9 BSE cases were detected through these surveillance measures (http://www.maff.go.jp/soshiki/seisan/bse/bse-i.htm).

These 27 BSE cases found by screening at slaughterhouses and by surveillance on farms were classified into five groups, depending on the year of birth as shown in Table 2. None of these cases showed clinical signs specific of BSE besides symptoms such as dislocation. Two cases (case 8: a 23-month-old steer, case 24: a 169-month-old beef cow) showed Western blot patterns different from those of the other BSE cases, indicating atypical cases [12].

Table 2. Twenty-seven cases of bovine spongiform encephalopathy classified depending on the year of birth

Year of birth	Cases	
	Screening	Surveillance
Before 1995	24	
1995, 1996*	2, 3, 4, 5, 6, 7, 10, 13, 16, 19	1, 11, 15
1999	12, 18	
2000	20, 23	14, 17, 21, 22, 26, 27
2001**, 2002	8, 9, 25	

MBM meat-and-bone meal.
* 1996: notification of MBM ban.
** 2001 complete MBM ban by law.

RISK FACTORS OF BSE IN JAPAN

Outcome of epidemiological investigations into BSE in Japan

In September 2002, MAFF convened a BSE epidemiological study group within the Ministry's BSE Technical Committee to investigate the source of infection and routes of transmission for the seven BSE cases that had been confirmed at that time.

The following risk factors were considered as the principal objective of the investigation: imports of live cattle, imports of MBM and of animal fat used as milk replacer.

Import statistics were analysed for all countries of origin where BSE had been reported at that time. In the report published in September 2003 [7], the countries of origin of potential BSE-infectious materials or live cattle as the

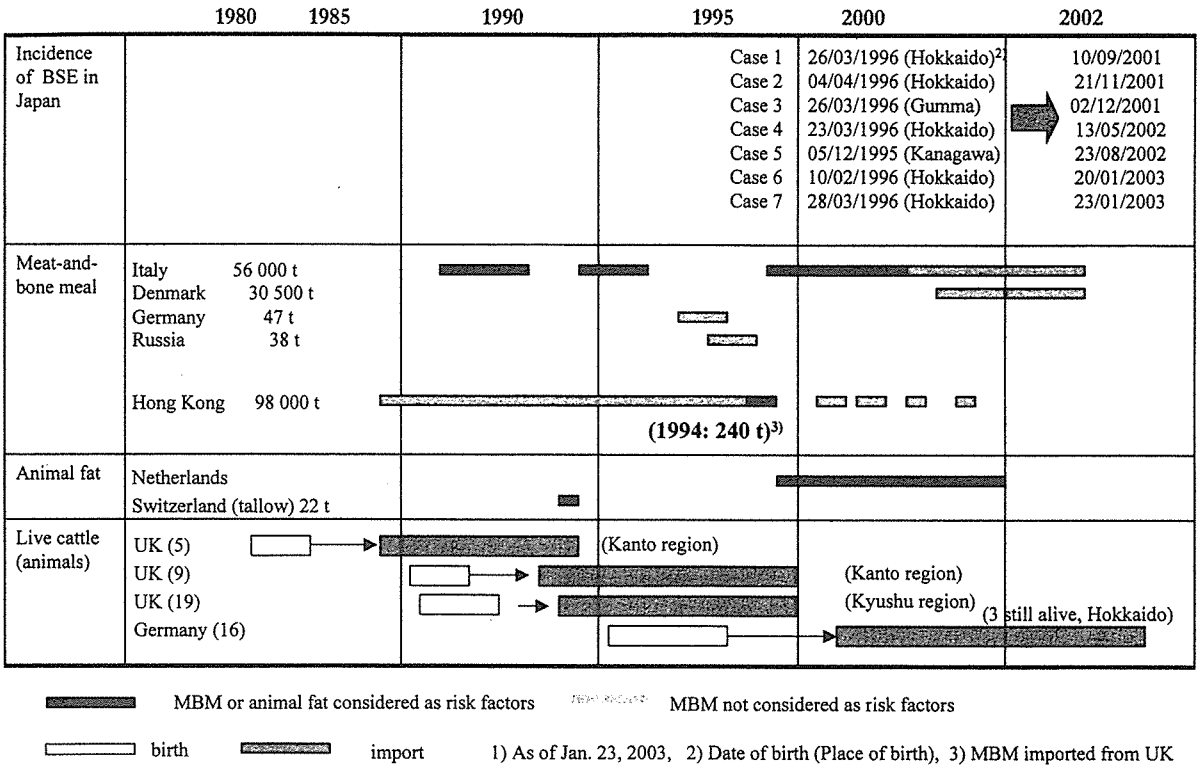

Fig. 1. Investigation into the source of infection of seven BSE cases[1].

potential infection source are listed and shown in Fig. 1.

Source of infection

(1) It is reasonable to assume that the BSE agent originated in the United Kingdom and was imported from there, either directly or indirectly. In this respect, candidates for infection were live cattle (from the Canada, Germany and the United Kingdom), MBM from Italy and animal fat from the Netherlands.

(2) It is possible that live cattle imported from the United Kingdom in 1982 or 1987 included BSE-infected animals. These cattle may have eventually contributed to a load of BSE-infectivity circulating in Japanese cattle feeds, after they had been slaughtered and their slaughter by-products rendered as MBM. Finally these potential sources of infection could have been recycled and amplified in the Japanese cattle population, becoming the source of infection for further cattle at risk.

(3) The live cattle imported from Germany are unlikely to be the source of infection, considering the timing of BSE incidence in Japan.

(4) It is considered highly unlikely that the live cattle imported from Canada were the source. However, relevant data are limited at present and it may be necessary to re-examine this information depending on future findings.

(5) The possibility cannot be ruled out that MBM imported from Italy before 1990 contained the BSE agent, that domestic cattle were exposed to this and were subsequently processed as MBM after slaughter, dismembering and rendering, and that they then became the source of infection.

(6) The animal fat imported from the Netherlands is unlikely to have contained animal protein and is therefore thought unlikely to have been contaminated with the BSE agent. Although we cannot ignore the fact that this animal fat is common to all seven cases in Japan to date, the results of our hypothesis-verification, case-control study and quantitative risk assessment revealed no indication that any of the seven cases could be linked directly to this potential source of infection.

Infection routes

Of the infection sources outlined above, the infection route through MBM is inconceivable through direct feeding, since none of the affected farms fed MBM to their cattle. However, BSE-positive cases were discovered in which feed for cattle, swine and poultry shared the same production lines in compound feed factories. These could have caused cross-contamination of compound feed for cattle during manufacturing or shipping. Considering a large number of cases of animals BAB (born after the ban) in the

United Kingdom, it is thought highly likely that infection in Japan was also caused by cross-contamination.

Animal fat was regularly added as an ingredient in milk replacers and could have been an indirect or secondary source of infection if it had been cross-contaminated with the BSE agent. As stated above, however, there is no proof or indication that any of the seven cases could be linked directly to this potential source of infection.

Scrapie as a potential source of infection in Japan

The number of sheep and goats in Japan is only 10,000 sheep and 21,000 goats.

In Japan, scrapie was first identified in 1984. Since then, a total of 63 cases have been confirmed, all of which were in sheep.

Since May 2001, surveillance for scrapie has been conducted at the level of slaughterhouses; sheep over 24 months of age (and over 12 months since April 2004) using the Western blot method and later the ELISA. By June 2004, approximately 600 sheep had been tested, but no scrapie-positive cases were detected. As the number of sheep is so low, BSE risk analysis has not been conducted.

SURVEILLANCE

Active surveillance was initiated in April 2001 by testing risk animals. In September 2001, surveillance was enhanced and a notification was issued enforcing compulsory testing and incineration of cattle displaying central nervous system symptoms. However, the surveillance of all animals at risk was a lengthy process as it was necessary to set up stock points to maintain fallen stock in refrigeration prior to testing and to separate the rendering facility that receives only carcasses of BSE-positive cattle.

There was an issue that carcasses had been left for a long period in poor storage conditions in some cases. Consequently, the use of the Western blot technique and immunohistochemistry were used on autolysed and/or deteriorated samples in simulated conditions [1, 14].

As the nationwide testing system of animals at risk was completed in April 2004, testing has become mandatory for all cattle that have died over 24 months of age. In accordance with these measures, by the end of March 2006, BSE testing had been conducted on a total of 247,781 animals at risk: 1,095 animals in 2001; 4,315 animals in 2002; 48,411 animals in 2003; 98,650 animals in 2004; and 95,310 animals in 2005. As a result, in addition to the first confirmed case of BSE, BSE testing revealed eight additional cases by May 2006. All of these cows were incinerated. Although the use of MBM in feed has been banned in Japan since October 2001, the delay in the commencement of testing animals at risk made it difficult to grasp the true status of BSE contamination in Japan.

The annual BSE incidence rate per million of animals over 24 months of age was 1.44 in 2001, 0.97 in 2002, 1.96 in 2003 and 2.49 in 2004. In 2004, the positive ratio of BSE through active monitoring was 0.0004%, whereas that with passive surveillance could not be calculated.

CONTROL MEASURES IN CATTLE

Since October 2001, several measures to prevent BSE spread in cattle have been implemented. They include the following:

(1) a complete ban on the use of MBM in feed for all animals
(2) incineration of all SRM
(3) incineration of all carcasses and by-products of confirmed BSE cases and of BSE-positive tested cattle at slaughterhouses
(4) a ban on MBM imports from all countries.

Few tests have been conducted on feed produced in Japan to determine whether MBM had been mixed into the feed (724 tests were conducted between October 2001 and the end of March 2004). MBM has been detected in only one case: in February 2005, protein derived from poultry was detected in cattle feed at a compound feed plant where cattle feed and poultry/pig feed containing chicken meal were produced on the same production line.

Japan relies on imports for approximately 90% of its concentrate feed, such as feed grains, which are used as raw materials in the domestic production of compound and mixed feed. Although imports consist mostly of single-ingredient feed, such as grain, compound feed and mixed feed manufactured overseas are also imported, but to a minor extent (<0.5% of the total amount used in Japan). As the true situation with regard to BSE contamination in exporting countries is unclear, there is no way of predicting whether it is possible to prevent compound or mixed feed that has been adulterated with MBM from being imported into the country. Consequently, the ingredients that are included in compound or mixed feed will be added to the items for which feed importers must submit notification.

A decision was taken in June 2003 to separate compound feed production lines for cattle from MBM production lines in rendering plants. This was implemented fully in April 2005. Carcasses of animals at risk subjected to active surveillance are disposed of in a separate rendering facility and the resulting MBM is incinerated.

The cattle traceability system was initiated in January 2002. In December 2003, traceability was made mandatory in the production stage, and subsequently in the market distribution stage in December 2004. This system enabled identification of each cow born in or after July 2003.

PUBLIC HEALTH MEASURES AT SLAUGHTERHOUSES

Cattle

Since October 2001, blanket BSE testing has been implemented at slaughterhouses at both prefecture and city government levels. The test is performed by meat inspectors who are civil servants and have a veterinarian licence. In

April 2005, testing was changed to cattle over 21 months of age. However, blanket BSE testing will continue for an additional period of three years and will be subsidised by the government for reasons of consumer confidence.

Removal of SRM (head, excluding the tongue and cheek flesh, spinal cord, distal ileum) has been mandatory since October 2001. Removal of the tonsil was added in October 2002 and the removal of the vertebral column containing the dorsal root ganglion in February 2004.

Accumulation of relatively small amounts of abnormal prion protein was found in the peripheral nerves and adrenal glands by the Western blot method in some BSE-positive animals that were subjected to a comprehensive examination. As these tissues are not listed in SRM, a further study on the distribution of BSE prion in these tissues is ongoing [2, 3].

The use of a spinal cord suction apparatus to remove the spinal cord prior to the splitting of carcasses was introduced gradually and is now used in 90% of slaughterhouses.

As of January 2005, there are 6 facilities of a total of 160 slaughterhouses that do not perform carcass splitting. Of the 154 facilities in which carcass splitting is performed, nearly 100% (from 99.4% to 100%) have implemented techniques to prevent the spread of spinal cord tissue on the carcass. Furthermore, there are 125 facilities (91.9%) that conduct suction removal of the spinal cord prior to carcass splitting. The removal by suction of spinal cord tissue is effective in 52.5% to 99.1% of cases, but washing the dressed carcass and removing the spinal cord dura matter after splitting results in the carcass being 100% free of any visible contamination with spinal cord fragments. However, tests detecting a marker protein contained in the spinal cord, glia fibrillary acidic protein (GFAP) have, in some cases, shown minute traces of GFAP on the surface of the lower portion of the carcass. The absence of spinal cord fragments after splitting is confirmed visually by meat inspectors on each animal. All saws, knives and other utensils are washed and disinfected after the slaughter of each animal. Furthermore, the removal of the spinal cord and washing of the carcass greatly reduces the risk of BSE contamination of the meat [8].

The pithing procedure during the slaughtering process is used in approximately 70% of all slaughterhouses in Japan (used on an estimated 80% of all slaughtered cattle). Pithing involves inserting a wire-like instrument into the head of the stunned animal to destroy the spinal nerve tissue, thereby preventing kicking reflexes by the animal. As the MHLW does not yet have sufficient grounds to ban this procedure, the Ministry has not yet announced any measures to ban pithing.

At the beginning, when a series of control measures were implemented, removal of SRM, together with the procedures at slaughter were not sufficient to protect human health. This is shown by the fact that the vertebral column was not included into the SRM list and that the spinal cord was not removed before splitting. We consider that such a risk, associated with incomplete removal of SRM and possible contamination of meat with SRM during slaughter, was overcome by blanket BSE testing as the cattle confirmed BSE-positive were removed from the food chain. As the traceability system was not available at that time, the adoption of blanket BSE testing was also effective in regaining consumer confidence.

Sheep and goats

The removal of the head, excluding the tongue and cheek flesh, spinal cord, tonsil, spleen and intestine of all sheep and goats at slaughterhouses was implemented in April 2002.

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