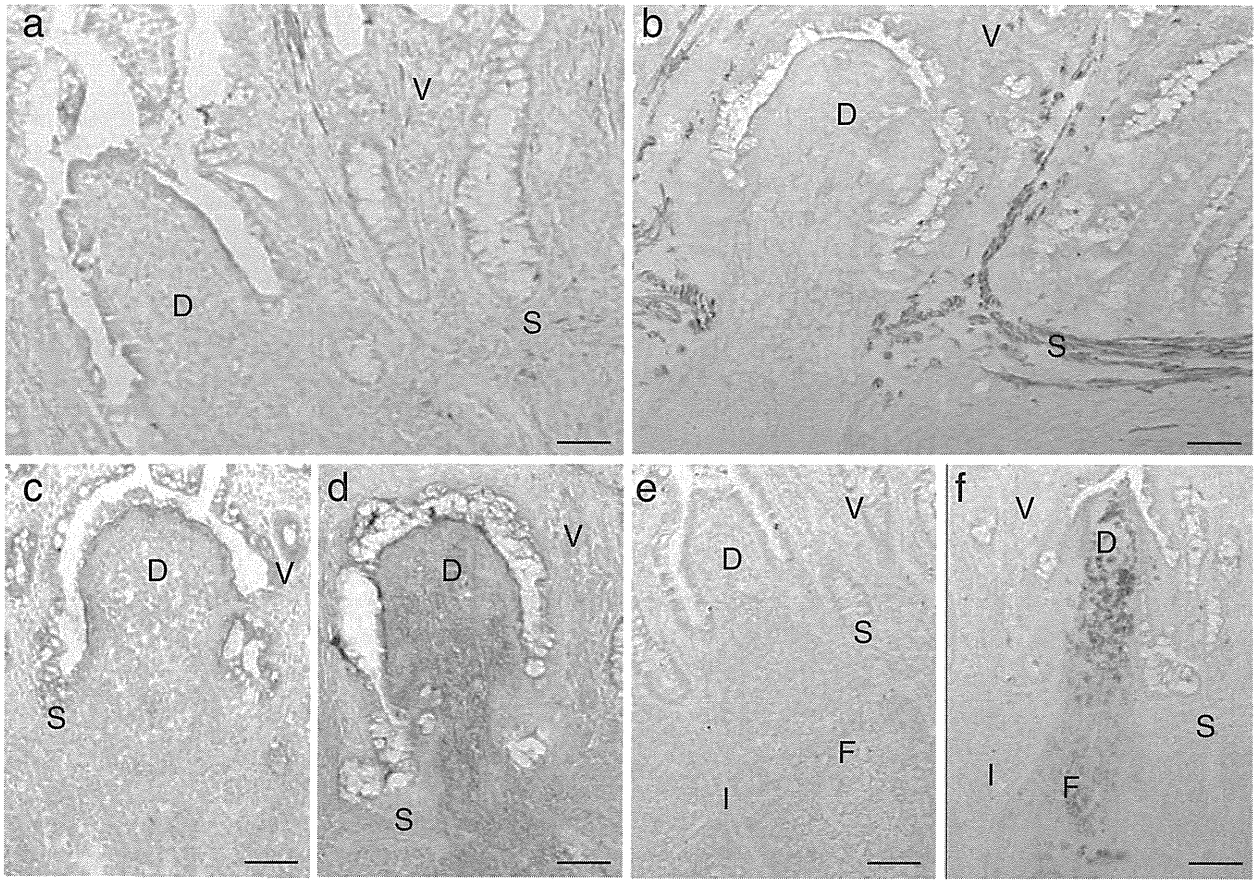

Fig. 3 Expression of ILR in the ileal Peyer's patches of newborn and 2-month-old calves (*D* dome, *S* submucosa, *F* follicles, *V* villous region). **a** CD25 expression was detected in the FAE, villous region, and submucosa in the ileal PP of newborn calves. **b** In 2-month-old calves, strong expression of $CD25^+$ cells was found in the villous region and submucosa. $IL\text{-}4R^+$ cells were more prominent in the dome region of 2-month-old calves (**d**) than in newborn calves (**c**). Few $IL\text{-}13R^+$ cells were found in the dome region and follicles of newborn calves (**e**), but many $IL\text{-}13R^+$ cells were present in 2-month-old calves (**f**). *Bars* 100 μm (**a**–**f**)

calves, few $IL\text{-}4R^+$ and $IL\text{-}13R^+$ cells are observed in newborn calves. IL-4 and IL-13 are the key cytokines in the pathogenesis of allergic inflammatory disease (Andrews et al. 2006). Two distinct IL-4 receptors occur: type 1, which includes IL-4Rα and CD132 (IL-2Rα), and type 2, which includes IL-4Rα and IL-13Rα1 (Ratthé et al. 2009). IL-13R also possesses two types of receptors: the heterodimers, composed of the IL-13Rα1 chain and IL-4Rα chain, or IL-13Rα2 (Arima et al. 2005). IL-4 and IL-13 have many functional properties in common because of a common receptor complex comprising IL-4Rα and IL-13Rα1 (LaPorte et al. 2008). In this study, polyclonal antibodies against IL-4Rα and IL-13Rα2, which has a high affinity for IL-13 but not IL-4, have been used; therefore, the distributions of $IL\text{-}4R^+$ and $IL\text{-}13R^+$ cells are remarkable. IL-4 has anti-inflammatory functions and proinflammatory functions. For instance, IL-4 induces the production of an IL-1R antagonist considered to be an anti-inflammatory cytokine that blocks IL-1 effects in neutrophils and the recruitment of inflammatory cells by increasing the expression of vascular cell adhesion molecule-1 on the endothelial surface (Ratthé et al. 2009). Although the effect of IL-13 binding to IL-13Rα2 is unclear, IL-13 plays a central role in host defense against parasite infection and in the pathogenesis of allergic diseases by binding to IL-4Rα and IL-13α1 (Andrews et al. 2009). Thus, the results of this study showing the expression of IL-4R and IL-13R indicate that several immune responses, such as the above-mentioned activity, might occur during the newborn to 2-month-old period, especially in the dome region in which immune responses are pronounced. On the other hand, few differences exist in the expression of IL-6R and IL-10R in the ileal PPs of newborn and 2-month-old calves. $IL\text{-}6R^+$ and $IL\text{-}10R^+$ cells have been widely detected in several areas of the PPs of newborn calves. These data suggest that no marked immunological changes occur with regard to IL-6R and IL-10R, at least during the 2 months

immediately after birth. Further, IL-6R and IL-10R might have been expressed in the prenatal period.

In conclusion, although the number of cattle examined is small for evaluations, the present study demonstrates that the distribution of the immune cells in ileal PPs differs between newborn and 2-month-old calves. Moreover, our results also indicate that the expression of ILRs markedly change from the newborn stage to 2 months of age. These data are the first evidence of the remarkable development of cytokine receptors in the ileal PPs of newborn calves and provide a basis for further investigation of the immature immune system in newborn calves and the dramatic development of the immune system in ileal PPs. Furthermore, in view of the high risk of infection in newborn calves, these results provide useful data for further studies on the interaction between the immune systems in the PPs of calves and infectious agents such as bacteria, viruses, and prions. Although immunohistochemical analysis has been carried out, the details of the expression of ILRs remain to be elucidated. Further studies will be required to define the expression and the function of various immune cells in the ileal PPs of calves.

References

Ackermann MR, Cheville NF, Deyoe BL (1988) Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Vet Pathol 25:28–35

Andrews AL, Holloway JW, Holgate ST, Davies DE (2006) IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets. J Immunol 176:7456–7461

Andrews AL, Nordgren IK, Kirby I, Holloway JW, Holgate ST, Davies DE, Tavassoli A (2009) Cytoplasmic tail of IL-13Rα2 regulates IL-4 signal transduction. Biochem Soc Trans 37:873–876

Arima K, Sato K, Tanaka G, Kanaji S, Terada T, Honjo E, Kuroki R, Matsuo Y, Izuhara K (2005) Characterization of the interaction between interleukin-13 and interleukin-13 receptors. J Biol Chem 280:24915–24922

Azzali G (2003) Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. Immunol Rev 195:178–189

Bayer AL, Yu A, Malek TR (2007) Function of the IL-2R for thymic and peripheral CD4+ CD25+ Foxp3+ T regulatory cells. J Immunol 178:4062–4071

Burchill MA, Yang J, Vang KB, Farrar MA (2007) Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunol Lett 114:1–8

David CW, Norrman J, Hammon HM, Davis WC, Blum JW (2003) Cell proliferation, apotosis, and B- and T-lymphocytes in Peyer's patches of the ileum, in thymus and in lymph nodes of preterm calves, and in full-term calves at birth and on day 5 of life. J Dairy Sci 86:3321–3329

Donovan GA, Dohoo IR, Montgomery DM, Bennett FL (1998) Calf and disease factors affecting growth in female Holstein calves in Florida, USA. Prev Vet Med 33:1–10

Evans CW, Lund BT, McConnell I, Bujdoso R (1994) Antigen recognition and activation of ovine γδ T cells. Immunology 82:229–237

Fotopoulos G, Harari A, Michetti P, Trono D, Pantaleo G, Kraehenbuhl JP (2002) Transepithelial transport of HIV-1 by M cells is receptor-mediated. Proc Natl Acad Sci USA 99:9410–9414

Landsverk T (1981) Peyer's patches and the follicle-associated epithelium in diarrheic calves. Pathomorphology, morphometry and acid phosphatase histochemistry. Acta Vet Scand 22:459–471

LaPorte SL, Juo ZS, Vaclavikova J, Colf LA, Qi X, Heller NM, Keegan AD, Garcia KC (2008) Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. Cell 132:259–272

Liebler-Tenorio EM, Riedel-Caspari G, Pohlenz JF (2002) Uptake of colostral leukocytes in the intestinal tract of newborn calves. Vet Immunol Immunopathol 85:33–40

Lwin S, Inoshima Y, Atoji Y, Ueno H, Ishiguro N (2009a) Immune cell types involved in early uptake and transport of recombinant mouse prion protein in Peyer's patches of calves. Cell Tissue Res 338:343–354

Lwin S, Inoshima Y, Ueno H, Ishiguro N (2009b) Uptake and transport of foreign particles in Peyer's patches of both distal ileum and jejunum of calves. Cell Tissue Res 337:125–135

Mackay CR, Hain WR (1989) A large proportion of bovine T cells express the γδT cell receptor and show a distinct tissue distribution and surface phenotype. Int Immunol 1:540–545

Malek TR, Porter BO, Codias EK, Scibelli P, Yu A (2000) Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature cells with severely impaired IL-2 receptors. J Immunol 164:2905–2914

Matsumoto S, Nanno M, Watanabe N, Miyashita M, Amasaki H, Suzuki K, Umesaki Y (1999) Physiological roles of γδ T-cell receptor intraepithelial lymphocytes in cytoproliferation and differentiation of mouse intestinal epithelial cells. Immunology 97:18–25

Naessens J, Howard CJ (1991) Monoclonal antibodies reacting with bovine B cells (BoWC3, BoWC4 and BoWC5). Vet Immunol Immunopathol 27:77–85

Naessens J, Olubayo RO, Davis WC, Hopkins J (1993) Cross-reactivity of workshop antibodies with cells from domestic and wild ruminants. Vet Immunol Immunopathol 39:283–290

O'Brien RL, Born WK (2010) γδ T cell subsets: a link between TCR and function? Semin Immunol 22:193–198

Paar M, Liebler EM, Pohlenz JF (1992) Uptake of ferritin by follicle-associated epithelium in the colon of calves. Vet Pathol 29:120–128

Parsons KR, Howard CJ, Jones BV, Sopp P (1989) Investigation of bovine gut associated lymphoid tissue (GALT) using monoclonal antibodies against bovine lymphocytes. Vet Pathol 26:396–408

Parsons KR, Bembridge G, Sopp P, Howard CJ (1993) Studies of monoclonal antibodies identifying two novel bovine lymphocyte antigen differentiation clusters: workshop cluster (WC) 6 and 7. Vet Immunol Immunopathol 39:187–192

Pithua P, Wells SJ, Godden SM, Raizman EA (2009) Clinical trial on type of calving pen and the risk of disease in Holstein calves during the first 90 d of life. Prev Vet Med 89:8–15

Ratthé C, Ennaciri J, Garcês Gonçalves DM, Chiasson S, Girard D (2009) Interleukin (IL)-4 induces leukocyte infiltration in vivo by an indirect mechanism. Mediators Inflamm 2009:193970

Sanglid PT, Fowden AL, Trahair JF (2000) How does the foetal gastrointestinal tract develop in preparation for enteral nutrition after birth? Livest Prod Sci 66:141–150

Soni J, Baird AW, O'Brien LM, McElroy M, Callanan JJ, Bassett HF, Campion D, Brayden DJ (2006) Rat, ovine and bovine Peyer's patches mounted in horizontal diffusion chambers display sampling function. J Control Release 28:68–77

Stone DM, Norton LK, Davis WC (1997) Modulation of bovine leukemia virus-associated spontaneous lymphocyte proliferation by monoclonal antibodies to lymphocyte surface molecules. Clin Immunol Immunopathol 83:156–164

Svensson C, Lundborg K, Emanuelson U, Olsson SO (2003) Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. Prev Vet Med 58:179–197

Urban EM, Chapoval AI, Pauza CD (2010) Repertoire development and the control of cytotoxic/effector function in human γδ T cells. Clin Dev Immunol 2010:732893

Yasuda M, Fujino M, Nasu T, Murakami T (2004) Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and development of IgG^+ and IgA^+ cells in lymphoid follicles. Dev Comp Immunol 28:357–369

Yasuda M, Jenne CN, Kennedy LJ, Reynolds JD (2006) The sheep and cattle Peyer's patch as a site of B-cell development. Vet Res 37:401–415

Amyloid, 2011; 18(3): 112-118
© 2011 Informa UK, Ltd.
ISSN 1350-6129 print/ISSN 1744-2818 online
DOI: 10.3109/13506129.2011.582901

informa
healthcare

RESEARCH ARTICLE

Pathogenesis of experimental amyloid protein A amyloidosis in sore hocks-affected rabbits

Tomoaki Murakami[1,2], Yasuo Inoshima[1,2], Ken-Ichi Watanabe[3], Yoshiyasu Kobayashi[1,3], Takane Matsui[1,3], Hisao Kurazono[1,4], & Naotaka Ishiguro[1,2]

[1]United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan [2]Laboratory of Food and Environmental Hygiene, Department of Veterinary Medicine, Gifu University, Gifu, Japan [3]Laboratory of Veterinary Pathology, Division of Pathological Science, Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan [4]Department of Applied Veterinary Medicine and Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan

Although the experimental transmission of amyloid protein A (AA) amyloidosis with amyloid-enhancing factor has been studied intensively, its pathogenesis remains obscure. We previously found that rabbits affected with 'sore hocks' (SH) uniquely developed AA amyloidosis in response to primary inflammatory stimulation followed by the administration of bovine AA fibrils. However, it is unknown why only the rabbits with preexisting SH developed experimental AA amyloidosis. There may be hidden factors in the SH status that stimulate the mechanism of cross-species transmission of AA amyloidosis. To examine the essential factors in the development of experimental AA amyloidosis in SH-affected rabbits, we studied the etiology of SH in rabbits pathologically and bacteriologically. In addition, we developed artificial SH symptoms in normal rabbits by use of an adjuvant prepared from *Staphylococcus aureus* (StA) isolated from a spontaneous SH-affected rabbit, and we evaluated the incidence of AA amyloidosis in rabbits with or without experimental SH symptoms. We found that StA administration was extremely efficient at stimulating the induction of experimental AA amyloidosis, and the influence of SH was required. We found that the persistent *S. aureus* infection in SH facilitates the development of experimental AA amyloidosis in rabbits and that the inflammatory stimulation provided by SH acts as an additional accelerator in experimental AA amyloidosis.

Keywords: AA amyloidosis, amyloid-enhancing factor, bovine amyloid fibril, rabbit, sore-hocks, Staphylococcus aureus

Abbreviations: AA, amyloid A; AEF, amyloid-enhancing factor; SAA, serum amyloid A; ELISA, enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ISA, iodine sulfuric acid; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBS, phosphate buffered saline; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; SED, staphylococcal enterotoxin D; SH, sore-hocks; StA, Staphylococcus spp. adjuvant

Introduction

Amyloidosis is a general term for several diseases that are characterized by the extracellular deposition of amyloid fibrils with a β-sheet structure in different organs and tissues [1]. Approximately 30 precursor proteins for amyloidosis that form amyloid fibrils in the human body have been identified; each precursor protein provokes one or a few disorders [2]. Amyloid protein A (AA) amyloidosis is a major form of systemic amyloidosis that is fatal. AA amyloidosis occurs in patients with rheumatoid arthritis or other chronic inflammatory diseases. The precursor protein of AA is serum amyloid A (SAA), which is an acute-phase reactant synthesized by the liver in response to inflammation [3]. At continually high concentrations, SAA can spontaneously aggregate into amyloid fibrils by an undetermined process [1].

AA amyloidosis can be induced in mice by repeated exposure to inflammatory stimulants, such as subcutaneous injections of silver nitrate, Freund's complete adjuvant (FCA), casein, or lipopolysaccharide (LPS) [4–7]. In these experimental systems, the systemic amyloidosis develops after a lag phase of several weeks. But, the lag phase can be dramatically shortened by administration of an amyloid-enhancing factor (AEF) [8–10]. It has been suggested that AA amyloid fibrils, immunoglobulin light-chain amyloid fibrils, and Alzheimer brain extracts can act as AEFs [11].

Correspondence: Naotaka Ishiguro, Department of Veterinary Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Tel: +81-58-293-2864. Fax: +81-58-293-2864. E-mail: ishiguna@gifu-u.ac.jp

Bovine AA amyloidosis is a fatal disease in cattle [12,13]. Typical symptoms of bovine AA amyloidosis are diarrhea and nephrotic syndrome. However, amyloid deposits in organs and tissues are sometimes encountered in cattle without these typical symptoms. In fact, amyloid deposits in the kidneys have been observed in unsymptomatic slaughtered cattle at an unexpected incidence [14,15]. Furthermore, the widespread distribution of amyloid deposits has been observed in several tissues including muscle in cattle diagnosed with AA amyloidosis [16].

AEFs can sometimes function as cross-seeding factors between animal species [17,18]. Oral administration of bovine amyloid fibril, which is derived from cattle with AA amyloidosis, accelerates the development of experimental murine amyloidosis [19]. We previously investigated whether bovine AA amyloid fibrils act as an AEF in rabbits. We found that amyloidosis in rabbits was induced by the intravenous administration of bovine AA amyloid in conjunction with multiple injections of an FCA–LPS emulsion [20]. All rabbits that developed amyloidosis had preexisting ulcerative pododermatitis, which is known as 'sore hocks' (SH). SH is a pressure-related condition in which the weight-bearing undersides of rabbits' feet develop suppurative pododermatitis caused by infectious pyogenic bacterium such as *Staphylococcus spp.* Particularly, severe cases of SH can progress to osteomyelitis and septicemia [21]. It remains unclear why only the rabbits with preexisting SH developed experimental amyloidosis in our previous study. We speculate that some factor or factors in SH stimulate the mechanism of cross-species transmission of AA amyloidosis.

In the present study, we sought to understand the relationship between SH and experimental rabbit amyloidosis. We examined the etiology of SH in rabbits pathologically and bacteriologically, and we evaluated the incidence of AA amyloidosis in rabbits with or without experimental SH symptoms. We found that the persistent bacterial infection in SH facilitates the development of experimental AA amyloidosis in rabbits, and the inflammatory stimulation provided by SH acts as an additional accelerator in the development of experimental AA amyloidosis.

Materials and methods

Macroscopic and histological examination of SH lesions

SH lesions were collected from five randomly selected rabbits that had spontaneously developed SH. The lesions were examined macroscopically and fixed in 15% neutral buffered formalin. Then, they were embedded in paraffin, cut into 2-μm sections, and stained with hematoxylin and eosin (H&E). The grading of SH lesion was determined by gross observation as follows: +, mild lesion; ++, moderate lesion frequently occurring in conjunction with abscesses in subcutaneous tissue; +++, severe lesion with multiple abscess formations.

Bacterial isolation from SH lesion

A portion of a lesion was obtained from a rabbit that had spontaneously developed SH and was diagnosed with AA amyloidosis. The tissue was homogenized with 3 ml of sterilized phosphate buffered saline (PBS). The suspension was plated onto mannitol salt agar (Nissui, Tokyo, Japan) and blood agar at serial dilutions to obtain the total number of bacteria. Colonies on the mannitol agar plates and blood agar plates were counted and used for further experiments. Ten colonies on the mannitol and blood plates were purified and routinely identified as *Staphylococcus spp.* Enterotoxin production was examined by using an SET-RPLA kit (Denkaseiken, Tokyo, Japan).

Animals

Sixty-seven outbred, Japanese white rabbits weighing 2.1–4.3 kg were obtained from Japan-Ram (Hiroshima, Japan). At the time of receipt, 36 rabbits were spontaneously affected with SH, while the remaining 31 rabbits appeared to be healthy. The rabbits were kept in cages and received *ad libitum* access to CR-3 pellets (CLEA Japan, Tokyo, Japan) and tap water.

Preparation of bovine amyloid fibrils

Bovine AA amyloid fibrils were extracted from the kidneys of Holstein-Friesian cattle that had developed systemic AA amyloidosis. Amyloid fibrils were extracted by using the method described by Pras et al. [22]. Briefly, kidneys were homogenized in 0.15 M NaCl and centrifuged at 46,600*g* for 20 min at 4°C (Optima L-100XP: Type 45Ti rotor, Beckman, CA, USA), and then the supernatant was discarded. This process was repeated until the absorbance of the supernatant at 280 nm became less than 0.02. The pellet was homogenized with cold distilled water and centrifuged at 46,600*g* for 20 min at 4°C, and the supernatant was collected. This step was repeated for a total of four times, and the supernatants from the repeated extractions were pooled and centrifuged at 100,000*g* for 1 h at 4°C. The pellets were stained with alkaline Congo red and examined by polarized light microscopy to confirm the presence of amyloid fibrils. The pellets containing amyloid fibrils were dissolved in distilled water at a concentration of 20 mg wet weight/ml, and the suspensions were stored at 4°C until use.

Production of SH-like lesions in rabbits

To produce artificial SH (SH-like) lesions in rabbits, the planter hair was artificially removed from 18 healthy rabbits that were reared in wire-floor cages to promote the development of pododermatitis. Within a few weeks, the rabbits developed ulcerated lesion on their soles. The lesions appeared the same as SH but there were no abscesses in the SH-like lesions, while some abscess formations were observed in the real sore hock lesions. Therefore, the SH-like lesion were thought to be less severe than the SH lesions. The grading of SH-like lesion was determined by gross observation as follows: +, mild lesion; ++, moderate lesion; +++, severe lesion.

Preparation of bacterial adjuvant

A *Staphylococcus spp.* colony was inoculated onto lysogeny broth agar plates. After incubation for 24 h at 37°C, the colonies on the plates were suspended in 20 ml of sterile PBS and centrifuged at 16,770*g* for 10 min (6900 high-speed centri-

fuge: RA-400 rotor, Kubota, Osaka, Japan). This process was repeated one more time. The cell pellets were suspended in 15 ml of sterile PBS and heated at 70°C for 30 min to inactivate viable cells. The heated bacterial cell suspension was centrifuged at 16,770*g* for 10 min. The centrifuged cell pellets were dehydrated for a day. A total of 2 mg of dried cells was suspended in 10 ml of a solution of Bayol F and Arlacel A (17:3), and the suspension was used as adjuvant (heat-killed *Staphylococcus spp.* adjuvant: StA). Because the isolated *Staphylococcus aureus* strain from SH lesion had been purified and repeatedly cultured in the plates, there was minimal chance of contamination of any amyloid fibrils in the StA.

Induction of rabbit amyloidosis

The experiments with rabbits were conducted essentially according to the protocol described by Horiuchi et al. [20]. All rabbits were subjected to a series of five intradermal injections with an interval of 4 days between each injection. Each injection contained two kinds of adjuvant. Rabbits in groups A, B, and C were inoculated with 1 ml of an FCA (Calbiochem, CA, USA) emulsion containing 200 μg of LPS derived from *Escherichia coli* O:111 B:4 (Wako, Tokyo, Japan). Rabbits in groups D, E, and F were inoculated with 1 ml of an StA–LPS emulsion. Rabbits in groups A and D were originally affected with SH, while rabbits in groups B and E had artificially produced SH. Rabbits in groups C and F, which did not have SH, were used as controls.

Rabbits in all groups (A to F) received an intravenous injection of 1 ml of bovine amyloid fibril solution, which was performed concurrently with the last inflammatory stimulation. The rabbits were sacrificed on the 2nd or 3rd day after bovine amyloid administration. Macroscopic, histological, and immunohistochemical examinations for rabbit amyloidosis were performed as described below. All animal experiment protocols were approved and performed according to the guidelines of the Committee for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine.

Measure of serum SAA concentration

The SAA concentrations in rabbit serum were measured by an enzyme-linked immunosorbent assay (ELISA) kit for multispecies SAA (BioSource International, Camarillo, CA, USA).

Rabbit sera were collected at the time of autopsy and stored at −20°C. At the time of measurement, the sera were thawed and diluted 1:500 with diluent buffer. ELISA was performed according to the manufacturer's instructions, and the serum concentration of SAA was expressed as absorbance values. To measure the normal SAA concentration in rabbits with or without SH, rabbit sera were collected from 12 healthy rabbits and nine rabbits with spontaneous SH before the first time of inflammatory stimulation.

Macroscopic, histological, and immunohistochemical examinations

The spleens and kidneys were collected from the rabbits. A portion of each organ was examined using routine methods with iodine sulfuric acid (ISA), and the remaining portions were fixed in 15% neutral buffered formalin, embedded in paraffin, cut into 2-μm sections, and stained with H&E or alkaline Congo red. The degree of amyloid deposits was determined by microscopic observation of H&E-stained tissue sections as follows: +, mild amyloid deposits; ++, moderate amyloid deposits; and +++, severe amyloid deposits. All amyloid deposits were checked by emerald green birefringence in sections stained with alkaline Congo red and exposed to polarized light. Immunohistochemical detection of amyloid deposits was performed with an Envision+ kit (Dako, Carpinteria, CA, USA) by using monoclonal mouse anti-human AA amyloid antibodies (1:200, Kyowa, Japan) as the primary antibody.

Results

Pathological and bacteriological examinations of SH-affected rabbits

Suppurative pododermatitis and subcutaneous abscesses were observed in all rabbits with SH. The epidermis of rabbits with SH was marked by ulceration and heavy crust formation. Histological examinations revealed inflammatory cellular infiltration and multifocal abscesses in the dermis. Multiple colonies of cocci were found in the center of some abscess.

To determine the etiological bacterium for rabbit SH tissue, a portion of SH tissue was suspended with PBS and inoculated onto mannitol salt agar plates and blood agar plates. A total number of $2.9–5.5 \times 10^8$ bacteria cells per gram of SH tissue was detected on both plates. From these results, the major bacterium in SH tissue appeared to be *Staphylococcus spp.* grown on mannitol salt agar plates. To identify the genus, five colonies on mannitol salt agar were examined by biochemical experiments. All colonies were Gram positive, coagulase positive, and catalase positive (data not shown), which suggested that they were *S. aureus*. An SET-RPLA kit was used to examine the enterotoxin production of five colonies. None of the colonies possessed enterotoxin genes such as SEA, SEB, SEC, or SED.

To examine the biological influence of *S. aureus* on the pathogenesis of rabbit AA amyloidosis, we produced adjuvant from *S. aureus* cell pellets to use as an inflammatory stimulator.

Induction of rabbit AA amyloidosis by bovine amyloid fibrils

The experimental induction of rabbit AA amyloidosis was performed as shown in Table I. Twenty-seven rabbits in group A and nine rabbits in group D possessed spontaneously occurring SH lesions, while 11 rabbits in group B and seven rabbits in group E had artificially induced SH (SH-like) lesions. A total of 44 rabbits in groups A, B, and C received injections of FCA, while 23 rabbits in groups D, E, and F received injections of StA.

Macroscopic observation of tissues exposed to ISA revealed that 7 of 27 (26%) rabbits in the group A, 5 of 9 (56%) rabbits in group D, and 2 of 7 (29%) rabbits in group E had amyloid deposits in their spleen and/or kidneys (Table I). Kidneys with amyloid deposits were typically larger and harder

than normal kidneys, and the color of the diseased kidneys was bleached out to ocher or brown. Similarly, spleens from rabbits with amyloid deposits were swollen and larger than normal spleens. Spleens from rabbits without amyloid deposits were also swollen due to extramedullary hematopoiesis. In ISA-exposed tissues, amyloid deposits in the spleen appeared blackish-brown and formed a leopard-print pattern (Figure 1A). Rabbits in group D (StA stimulation and carrying SH lesions) exhibited the highest incidence (56%) of amyloid deposits, as determined by ISA reaction. Rabbits without SH or SH-like lesions (groups C and F) had no amyloid deposits.

Measurement of serum SAA concentrations

To measure the serum SAA concentrations, we used an ELISA kit for multispecies SAA as shown for each group in Figure 2. In control, the serum SAA absorbance both in rabbits with and without SH was lower than in experimental groups. No significant differences in SAA values were observed between rabbits with and without amyloidosis in groups A, D, and E.

Histological examination of rabbit amyloidosis

The spleens and kidneys from all 67 rabbits were examined for amyloid deposits by histological and immunohistochemical analyses. Histological examinations revealed that 9 of 27 (33%) rabbits in group A, 7 of 9 (78%) rabbits in group D, and 3 of 7 (43%) rabbits in group E had amyloid deposits in the spleen and/or kidney (Table I). Amyloid deposits in five animals (two samples in group A, two samples in group D, and one sample in group E) were too slight to be detected by macroscopic observation with ISA reactant but could be observed by histological examinations. The severity of foot lesions and the degree of amyloid deposits appeared to be unrelated (Table II). Amyloid deposits in the spleen were mostly observed in the perifollicular area (Figures 1B and C). In kidneys, amyloid was deposited in the glomeruli at the cortex and the interstitium of the medulla and pelvis renalis (Figure 1D–F). No amyloid deposits were found in groups B, C, and F, as determined by H&E, Congo red, and immunohistochemistry (IHC) analyses. All amyloid deposits showing emerald

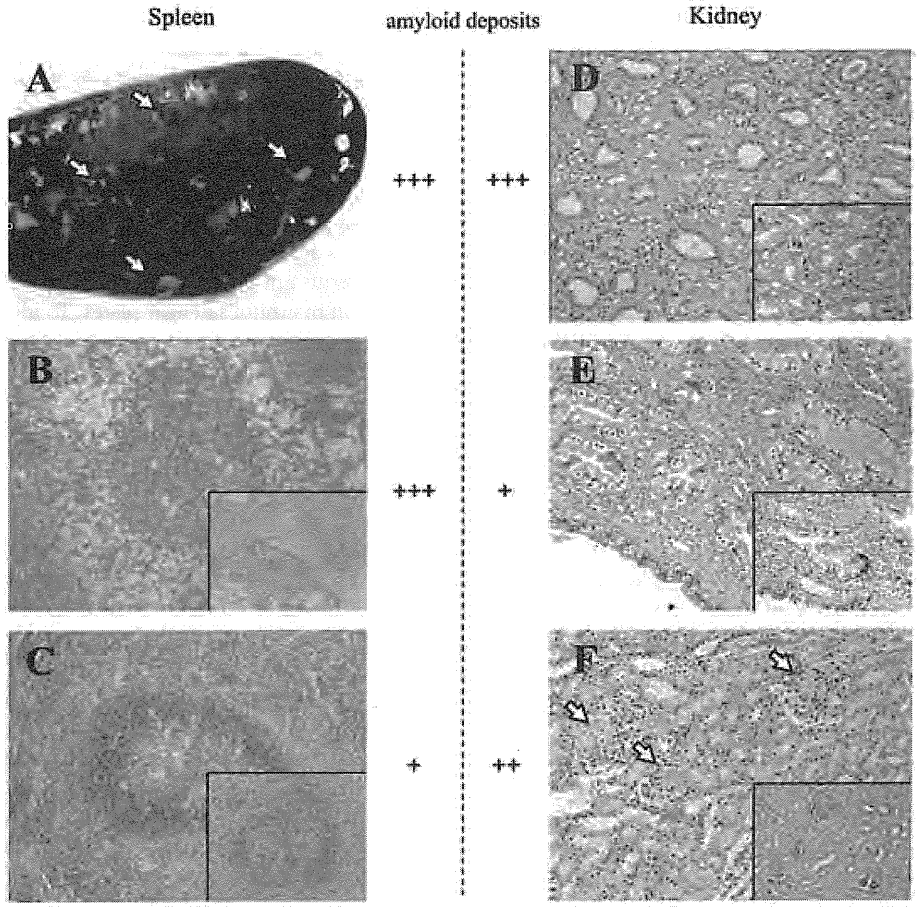

Figure 1. (A) In the ISA reaction, amyloid deposits were stained blackish-brown and formed a leopard-print pattern (arrows). (B) Amyloid deposits were found in the perifollicular area with Hematoxylin & eosin (H&E). The deposited amyloid was determined to be AA amyloid. (C) Amyloid deposits were not found with H&E, but a small amount of amyloid deposits was detected with anti-human AA amyloid mAb. Naturally, the amount of amyloid deposits could not be observed by macroscopic observation under ISA reactants. (D) Deposited amyloid was observed in the interstitium of the medulla in kidney (H&E). (E) Amyloid deposits were not found with H&E, but a small amount of amyloid deposits was detected with anti-human AA amyloid mAb. (F) Amyloid deposits were present in the glomeruli (arrows). Insets in B to F: Immunohistological staining with a 1:200 dilution of anti-human AA amyloid mouse monoclonal antibody. The degree of amyloid deposits in the tissues was indicated by +++: severe deposits, ++: moderate deposits, and +: mild deposits.

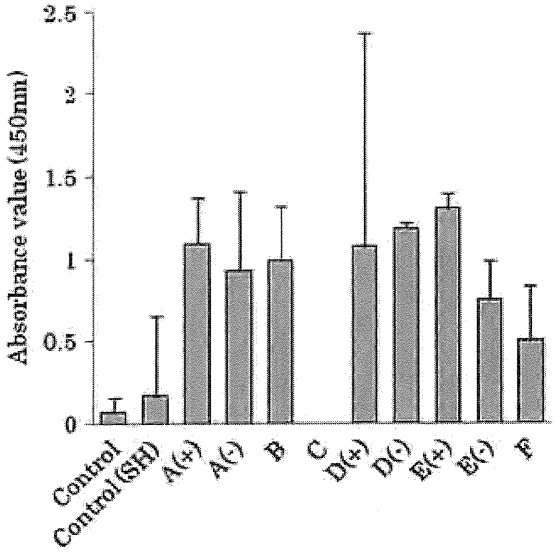

Figure 2. Serum SAA concentrations in each group rabbits. The bars indicate the median values. 'A (+), D (+), or E (+)' indicates rabbits with amyloid deposits in group A, D, or F, and 'A (−), D (−), or E (−)' indicates rabbits without amyloid deposits in group A, D, or F. The SAA concentrations collected from healthy rabbits (control) and SH rabbits (control SH) before the first inflammatory stimulation were 0.065 and 0.162 at a median, respectively. In group C, serum of rabbits could not be collected in this experiment.

green birefringence under polarized light were IHC-positive (Figure 1B,C).

Discussion

Based on the results of these experiments, it appears that SH plays a crucial role in the development of rabbit AA amyloidosis and that artificial SH (SH-like) is less effective than spontaneous SH in promoting the development of AA amyloidosis. Thus, there seems to be a difference in the stimulation provided by spontaneous SH and artificial SH. There was morphologically no abscess in the SH-like lesion, while some abscess formations were observed in the real sore hock lesion. Therefore, bacterial infection such as *S. aureus* found in spontaneous SH lesion might not be observed in SH-like lesion. Group D (SH and StA stimulations) showed the highest incidence of rabbit amyloidosis, suggesting that StA is a more efficient inducer of amyloidosis than FCA. This result is supported by a comparison of the incidence of amyloid deposits in groups E and B. Thus, StA may be an extremely efficient stimulator in the induction of rabbit amyloidosis. However, the StA is not only enough for induction of rabbit amyloidosis alone, but the continuous stimulation such as SH-like condition is need for establishment of amyloid deposits. Further studies of the influence of SH or the SH-like condition are required.

We isolated *S. aureus* from spontaneous SH in rabbits. The major problem in rabbit staphylococcosis is caused by high-virulence *S. aureus* strains [21]. However, the strain isolated in the present study did not possess enterotoxin genes such as SEA, SEB, SEC, and SED. Furthermore, StA used as an adjuvant was made from a strain that had been heat killed. On the other hand, the rabbits in groups D and E developed AA amyloidosis more frequently than the rabbits in groups A and B. From these results, we conclude that the amyloidogenesis of rabbit AA amyloidosis is not influenced by the direct action of toxins; rather, it is influenced by the indirect secondary action of *S. aureus* infection. In the present study, therefore, we deduce that somatic antigen of *S. aureus* cells stimulated the host immune system leading to the development of AA amyloidosis. In some species, spontaneous amyloidosis is often influenced secondary from bacterial infection with inflammatory reaction [12]. It is striking that waterfowl develop amyloidosis associated with *S. aureus* infection in 'bumblefoot' [23,24].

Table I. Experimental groups and number of rabbits with amyloidosis.

Group	Inflammatory stimulations	State of foot	Bovine amyloid[§]	No. of animals used	No. of rabbits with amyloidosis: ISA reaction	No. of rabbits with amyloidosis: Histological exam
A	FCA*	SH[‡]	+	27	7 (26%)	9 (33%)
B	FCA	SH-like	+	11	0	0
C	FCA	Normal	+	6	0	0
D	StA†	SH	+	9	5 (56%)	7 (78%)
E	StA	SH-like	+	7	2 (29%)	3 (43%)
F	StA	Normal	+	7	0	0

*FCA: Freund's complete adjuvant.
†StA: *Staphylococcus spp.* adjuvant.
‡SH: sore hocks. SH-like: artificially developed pododermatitis.
§Bovine amyloid: administration of 1 ml bovine amyloid fibril solution.

Table II. The severity of foot lesions and degree of amyloid deposits.

Group (SH or SH-like)	No. of rabbit	Grading of foot lesion*	Degree of amyloid deposits†: Renal medulla	Renal cortex	Spleen
Group A (SH)	1	+	++	−	++
	6	+	−	+	+
	17	++	++	+	−
	10	++	−	−	+++
	3	+++	−	−	+
	7	+++	++	−	+
	12	+++	++	++	+
	24	+++	+	+	++
	25	+++	−	−	+++
	3	+	+	+	−
	5	++	+	+	+
	7	++	+	+	++
Group D (SH)	2	+++	+	++	+++
	8	+++	−	+	++
	9	+++	−	−	++
	4	+++	+	+	+++
	2	+	−	−	++
Group E (SH-like)	7	+	−	−	++
	6	+++	+	−	−

*SH: +, mild legion; ++, moderate legion frequently associated with abscesses in subcutaneous tissue; +++, severe legion with multiple abscess formations SH-like: +, mild legion; ++, moderate legion; +++, severe legion.
†+, mild amyloid deposits; ++, moderate amyloid deposits; +++, severe amyloid deposits.

Bumblefoot is ulcerative pododermatitis with bacterial infection that occurs on the feet of birds, and its clinical condition mimics SH of rabbits. Pododermatitis, such as SH and bumblefoot, is hard to heal because the plantar tissues habitually sustain the bacterial infection. As a result, they provide persistent inflammatory stimulation and cause AA amyloidosis.

We observed elevated SAA absorbances in Figure 2, i.e. high plasma concentrations of SAA compared to the control groups. Amyloidogenic SAA can aggregate into amyloid fibrils at high plasma concentrations. Normally, it is believed that these aggregates are degraded by physiological homeostatic control [25]. However, after longstanding infectious or noninfectious inflammatory diseases with persistently high plasma concentrations of SAA, the degradation is sometimes insufficient, leading to AA amyloid deposits [1,26]. In several animals (including humans), there are certain disorders that occur with AA amyloidosis. Two examples are rheumatoid arthritis in humans and bumblefoot in waterfowl. These disorders trigger longstanding high plasma concentrations of SAA. However, few patients with these conditions develop AA amyloidosis [27,28]. In the present study, there was no significant difference in the SAA values of rabbits with and without amyloidosis (Figure 2). However, the results show that rabbits without foot lesions (group F) have the lowest average SAA concentration and that rabbits in group D, which showed the highest incidence of amyloid deposition, have the highest median SAA concentration of all the experimental groups. These results, even though not significant, suggest that SAA concentration influences the development of AA amyloidosis. Basically, it is thought that the high level of SAA influences the development of AA amyloidosis, but not directly. We conclude there must be other factors that influence the development of AA amyloidosis.

In the present study, we concluded that persistent *S. aureus* infection in SH as a significant factor to facilitate the risk of rabbit AA amyloidosis and that SH as an inflammatory mediator acts as an additional accelerator in rabbit AA amyloidosis. However, we also conclude that these experimental factors do not fulfill the condition of pathogenesis of AA amyloidosis completely, because not all rabbits in each group developed AA amyloidosis. The additional factors involved in the pathogenesis of AA amyloidosis are still unknown [25,29].

As shown in the results, bovine AA amyloid possesses transmissibility to rabbits. However, the transmission of bovine AA amyloid to rabbits in this experiment was less efficient than reported transmission to mice [18,19], and previously reported transmission to rabbits [20]. We hypothesize that the different rate of transmission between this experiment and previous experiments was caused by the variant severity of SH lesions, and also note that the transmission efficiency of bovine AA amyloid as AEF may be influenced by the recipient ability of the host species. For studies on the pathogenesis of AA amyloidosis, it is necessary to analyze the propagation factors for amyloidosis *in vivo*. The results of the present study suggest that the factors affecting the pathogenesis of experimental rabbit AA amyloidosis are both extraneous stimulations and host factors. Further investigations of the pathogenesis of AA amyloidosis must analyze the influence of the immunological state of patients.

Acknowledgment

We thank Mr. Maruyama for caring for the rabbits.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by a grant from the Intractable Disease Division, the Ministry of Health and Welfare, a Research Committee for Epochal Diagnosis and Treatment of Amyloidosis in Japan, and supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (Project Code: 00109521), Japan.

References

1. Merlini G, Bellotti V. Molecular mechanisms of amyloidosis. N Engl J Med 2003;349:583–596.
2. Westermark P, Benson MD, Buxbaum JN, Cohen AS, Frangione B, Ikeda S, Masters CL, Merlini G, Saraiva MJ, Sipe JD. A primer of amyloid nomenclature. Amyloid 2007;14:179–183.
3. Cray C, Zaias J, Altman NH. Acute phase response in animals: a review. Comp Med 2009;59:517–526.
4. Skinner M, Shirahama T, Benson MD, Cohen AS. Murine amyloid protein AA in casein-induced experimental amyloidosis. Lab Invest 1977;36:420–427.
5. Janigan DT, Druet RL. Experimental amyloidosis. Role of antigenicity and rapid induction. Am J Pathol 1966;48:1013–1025.
6. McAdam KP, Sipe JD. Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. J Exp Med 1976;144:1121–1127.
7. Ram JS, Glener GG, DeLellis RA. Amyloid. I. Use of Freund's adjuvant in experimental amyloidosis. Proc Soc Exp Biol Med 1968;127:854–856.
8. Axelrad MA, Kisilevsky R, Willmer J, Chen SJ, Skinner M. Further characterization of amyloid-enhancing factor. Lab Invest 1982;47:139–146.
9. Kisilevsky R, Gruys E, Shirahama T. Does amyloid enhancing factor (AEF) exits? Is AEF a single biological entity? Amyloid 1995;2:128–133.
10. Johan K, Westermark G, Engström U, Gustavsson A, Hultman P, Westermark P. Acceleration of amyloid protein A amyloidosis by amyloid-like synthetic fibrils. Proc Natl Acad Sci U S A 1998;95:2558–2563.
11. Cui D, Kawano H, Takahashi M, Hoshii Y, Setoguchi M, Gondo T, Ishihara T. Acceleration of murine AA amyloidosis by oral administration of amyloid fibrils extracted from different species. Pathol Int 2002;52:40–45.
12. Jakob W. Spontaneous amyloidosis of mammals. Vet Pathol 1971;8:292–306.
13. Takahashi E, Kuwayama H, Kawamoto K, Matsui T, Inokuma H. Detection of serum amyloid A isoforms in cattle. J Vet Diagn Invest 2009;21:874–877.
14. Fujinaga Y. Incidence of amyloid deposits in various organs in senile cows and their pathological characteristics [in Japanese with English abstract]. Yamaguchi Med J 1990;39:293–303.
15. Tojo K, Tokuda T, Hoshii Y, Fu X, Higuchi K, Matsui T, Kametani F, Ikeda S. Unexpectedly high incidence of visceral AA-amyloidosis in slaughtered cattle in Japan. Amyloid 2005;12:103–108.
16. Yamada M, Kotani Y, Nakamura K, Kobayashi Y, Horiuchi N, Doi T, Suzuki S, Sato N, Kanno T, Matsui T. Immunohistochemical distribution of amyloid deposits in 25 cows diagnosed with systemic AA amyloidosis. J Vet Med Sci 2006;68:725–729.
17. Lundmark K, Westermark GT, Nyström S, Murphy CL, Solomon A, Westermark P. Transmissibility of systemic amyloidosis by a prion-like mechanism. Proc Natl Acad Sci U S A 2002;99:6979–6984.

18. Liu Y, Cui D, Hoshii Y, Kawano H, Une Y, Gondo T, Ishihara T. Induction of murine AA amyloidosis by various homogeneous amyloid fibrils and amyloid-like synthetic peptides. Scand J Immunol 2007;66:495–500.
19. Cui D, Kawano H, Hoshii Y, Liu Y, Ishihara T. Acceleration of murine AA amyloid deposition by bovine amyloid fibrils and tissue homogenates. Amyloid 2008;15:77–83.
20. Horiuchi N, Kotani Y, Koga M, Yamada M, Kobayashi Y, Matsui T. Experimental induction of amyloidosis by bovine amyloid fibrils in sore hock rabbits. Amyloid 2008;15:84–88.
21. Okerman L, Devriese LA, Maertens L, Okerman F, Godard C. Cutaneous staphylococcosis in rabbits. Vet Rec 1984;114:313–315.
22. Pras M, Schubert M, Zucker-Franklin D, Rimon A, Franklin EC. The characterization of soluble amyloid prepared in water. J Clin Invest 1968;47:924–933.
23. Dias JL, Montau RJ. Staphylococcosis in captive exotic waterfowl. Avian Path 1994;23:659–669.
24. Tanaka S, Dan C, Kawano H, Omoto M, Ishihara T. Pathological study on amyloidosis in *Cygnus olor* (mute swan) and other waterfowl. Med Mol Morphol 2008;41:99–108.
25. Westermark GT, Westermark P. Prion-like aggregates: infectious agent in human disease. Trends Mol Med 2010;16:501–507.
26. Röcken C, Shakespeare A. Pathology, diagnosis and pathogenesis of AA amyloidosis. Virchows Arch 2002;440:111–122.
27. Takahashi E, Uzuka Y, Tanabe S, Satoh M, Furuoka H. Serum amyloid A and haptoglobin levels in bovine amyloidosis. J Vet Med Sci 2007;69:321–323.
28. Obici L, Raimondi S, Lavatelli F, Bellotti V, Merlini G. Susceptibility to AA amyloidosis in rheumatic diseases: a critical overview. Arthritis Rheum 2009;61:1435–1440.
29. Pepys MB, Rademacher TW, Amatayakul-Chantler S, Williams P, Noble GE, Hutchinson WL, Hawkins PN, Nelson SR, Gallimore JR, Herbert J, et al. Human serum amyloid P component is an invariant constituent of amyloid deposits and has a uniquely homogeneous glycostructure. Proc Natl Acad Sci USA 1994;91:5602–5606.

Prion 5:3, 1-4; July/August/September 2011; © 2011 Landes Bioscience

Real-time quaking-induced conversion

A highly sensitive assay for prion detection

Ryuichiro Atarashi,[1,*] Kazunori Sano,[1,2] Katsuya Satoh[1] and Noriyuki Nishida[1,2]

[1]Department of Molecular Microbiology and Immunology; Graduate School of Biomedical Sciences; [2]Global COE Program; Nagasaki University; Nagasaki, Japan

We recently developed a new in vitro amplification technology, designated "real-time quaking-induced conversion (RT-QUIC)," for detection of the abnormal form of prion protein (PrP^{Sc}) in easily accessible specimens such as cerebrospinal fluid (CSF). After assessment of more than 200 CSF specimens from Japanese and Australian patients, we found no instance of a false positive, and more than 80% accuracy for the correct diagnosis of sporadic Creutzfeldt-Jakob disease (sCJD). Furthermore, the RT-QUIC can be applied to other prion diseases, including scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE), and is able to quantify prion seeding activity when combined with an end-point dilution of samples. These results indicate that the RT-QUIC, with its high sensitivity and specificity, will be of great use as an early, rapid and specific assay for prion diseases.

Key words: RT-QUIC, real-time quaking-induced conversion, prion, CJD, Creutzfeldt-Jakob disease, CSF, cerebrospinal fluid

Submitted: 06/10/11

Accepted: 06/28/11

DOI:

*Correspondence to: Ryuichiro Atarashi; Email: atarashi@nagasaki-u.ac.jp

Diagnosis of Creutzfeldt-Jakob Disease: The Current Situation

Human prion diseases, including Creutzfeldt-Jakob disease (CJD), are incurable neurodegenerative disorders characterized by progressive spongiform changes and the accumulation of abnormal prion protein (PrP^{Sc}) in the central nervous system.[1] The majority of CJD cases (approximately 85%) are sporadic in nature, but the remaining cases comprise genetic and infectious forms. Iatrogenic CJD is the consequence of inadvertent transmission during medical procedures in which sporadic CJD (sCJD)-contaminated tissues or explants (such as dura mater and pituitary hormones) or surgical instruments were used.[2] Variant CJD (vCJD) is primarily a zoonosis that arose from contamination of the human food chain by bovine spongiform encephalopathy (BSE), although secondary transmission of vCJD by blood transfusion has also been reported in reference 3. Hence, by adopting additional infection control measures as appropriate, an early and accurate diagnosis of CJD would help to lessen the possibility of iatrogenic transmission and lead the way to timely therapeutic interventions. However, the definitive ante-mortem confirmation of CJD currently requires the presence of typical neuropathology together with the demonstration of PrP^{Sc} in specimens obtained by biopsy, the practice of which is often precluded both by the invasiveness of the procedure and the risks it poses to medical care staff. Thus, the highly sensitive detection of PrP^{Sc} in accessible body fluids such as cerebrospinal fluid (CSF) and blood can be expected to constitute a most valuable means for the early and specific diagnosis of CJD. However, because the concentration of PrP^{Sc} in these specimens is likely to be very low, one of the most promising approaches would be to develop an efficient amplification of PrP^{Sc} in vitro.[4,5] Indeed, several assays, including protein misfolding cyclic amplification (PMCA),[6,7] the amyloid seeding assay (ASA),[8] and quaking-induced conversion (QUIC),[9,10] have previously been reported to permit the sensitive detection of PrP^{Sc} in animal and human brain specimens. Nonetheless, early attempts at

ultrasensitive PrP^{Sc} detection in accessible body fluids were unsuccessful in human prion diseases. For this reason, we initiated studies aimed at establishing a highly sensitive assay for the detection of prion in human CSF.

Establishment of Real-Time QUIC (RT-QUIC)

In the QUIC assays, soluble recombinant PrP (rPrP-sen) expressed in *E. coli* is used as a substrate to amplify the minute amounts of PrP^{Sc}. Using a dedicated shaker, the reaction is enhanced by vigorous intermittent shaking which induces rPrP-sen to aggregate and form fibrils.[9] One of the advantages in QUIC is that shaking/agitation can be performed more easily and consistently than sonication, which has the problem of varied delivery of vibrational energy to the samples. On the other hand, further improvement in the rapidity and practicality of this method was required in order for it to become useful in the diagnosis of prion diseases, because the initial standard format of QUIC (S-QUIC) required a time-consuming western blot. Thus, we combined QUIC technology with thioflavin T (ThT) fluorescence dye, to monitor amyloid formation, in order to minimize the time necessary for the detection of protease-resistant rPrP fibrils (rPrP-res). We first determined if the shaking of our fluorescence microplate reader could induce PrP^{Sc}-dependent rPrP-res ($rPrP\text{-}res^{Sc}$) formation in a buffer containing 0.05–0.1% SDS, as in the S-QUIC. Human rPrP-sen (rHuPrP-sen) and CJD brain homogenate (BH) were used as a substrate and a seed, respectively, for the QUIC reaction. Unexpectedly, we did not observe $rPrP\text{-}res^{Sc}$ formation or an elevation in the ThT fluorescence using our microplate reader (unpublished data). Our explanation for this is that the shaking power of the microplate reader was not strong enough to elicit the QUIC reactions in the presence of SDS. SDS tends to cause fibrils to stack and stabilize rPrP-res polymers as a result. In fact, we observed that fibrils formed in the presence of SDS were much larger and thicker than those formed in its absence. Taken together, sonication in PMCA or vigorous shaking in S-QUIC seems to be required as a means of fragmenting the rPrP-res polymers formed in the presence of SDS.

We then tested whether $rPrP\text{-}res^{Sc}$ formation was induced when guanidine-HCl (GdnHCl) was added, because it has been thought that GdnHCl was required for the conversion of PrP-sen to PrP-res in a cell-free system.[11] Somewhat unexpectedly, we observed $rPrP\text{-}res^{Sc}$ formation even in the absence of GdnHCl. In contrast, the negative control reactions without seed and in the absence of GdnHCl exhibited a marked delay in spontaneous rPrP-res ($rPrP\text{-}res^{spon}$) formation.[12] For this reason, use of a GdnHCl-free buffer can dramatically reduce the risk of false-positive reactions and enhance the sensitivity of the method.

Shaking/agitation is considered to cause several facilitatory effects on the QUIC reaction. One is that a partial unfolding of a portion of the rPrP-sen is induced by increasing the air-water interface through which a denaturing boundary between the hydrophobic air and hydrophilic water is formed.[13] Next, shaking/agitation enhances the interaction between rPrP-sen and PrP^{Sc}, and the fragmentation of rPrP-res polymers.[14] The energetic barrier of spontaneous fibril formation is likely to be higher than that of seed-dependent fibril formation or elongation, because spontaneous formation initially necessitates nucleation as the rate-limiting step.[15]

Meanwhile, the extent of the partial unfolding of rPrP-sen by shaking alone is assumed to be more heterogeneous than that in the presence of GdnHCl, probably because the air-water interfaces are unequally distributed in the reaction mixture (**Fig. 1**). In contrast, the addition of GdnHCl accelerates the nucleation rate, resulting in an increase in the rate of spontaneous fibril formation. Of note, we observed that there was an inverse correlation between the rate of rPrP-res formation and the concentration of rPrP-sen substrate.[12] It has been reported that the aggregation rate of several other proteins is inversely correlated with the concentration of substrate protein in a denaturant-free buffer with shaking.[16,17] Conversely, previous studies using cell-free conversion[18] and rPrP fibril formation,[19-21] respectively, in the presence of denaturant or at low pH, have shown that the rate of PrP-res formation was directly proportional to the PrP-sen concentration. This seeming contradiction can be explained again by the difference in the denaturation status of PrP-sen under various conditions. We hypothesized that heterogenous denaturation of the substrate protein in a denaturant-free buffer with shaking is a major cause of the inverse correlation (**Fig. 1**).

We examined the effect of pH, and the concentrations of rHuPrP-sen and salt, on QUIC reactions in a GdnHCl-free buffer. We found that the presence of NaCl is essential for rPrP-res formation and the sensitivity of this method was maximal at 500 mM NaCl at pH 7.[12] The requirement for NaCl in the formation of rPrP-res is compatible with previous studies, which have shown that salt is required for cell-free conversion in the absence of GdnHCl[22] and the maintenance of a protease-resistant PrP^{Sc} conformation.[23]

We named this new assay "real-time QUIC (RT-QUIC)" by analogy with real-time PCR. The RT-QUIC enabled us to measure up to 96 replicates at a time, obtain the results immediately, and is potentially safer than S-QUIC or PMCA because the prions are sealed within a 96-well plate throughout the entire procedure.

Application of RT-QUIC to Diagnostic Tests for Human Prion Diseases

CJD has been categorized into six molecular subtypes (MM1, MM2, MV1, MV2, VV1, VV2) on the basis of whether methionine (M) or valine (V) is present at codon 129 of the gene encoding prion protein, combined with the profile of PrP^{Sc} (type 1 or type 2).[24] We evaluated the detection limit of MM1- and MM2-sCJD brain homogenate using RT-QUIC. The minimum amount of PrP^{Sc} in the brains detectable by RT-QUIC was around ~1 fg (10^{-15} g). To determine the applicability of RT-QUIC in the clinical diagnosis of sCJD, we compared the RT-QUIC seeding activity in CSF samples from patients with sCJD and patients without sCJD but with other neurodegenerative diseases such as Alzheimer disease. We decided upon CSF as the specimen because CSF is routinely used in the assessment of many

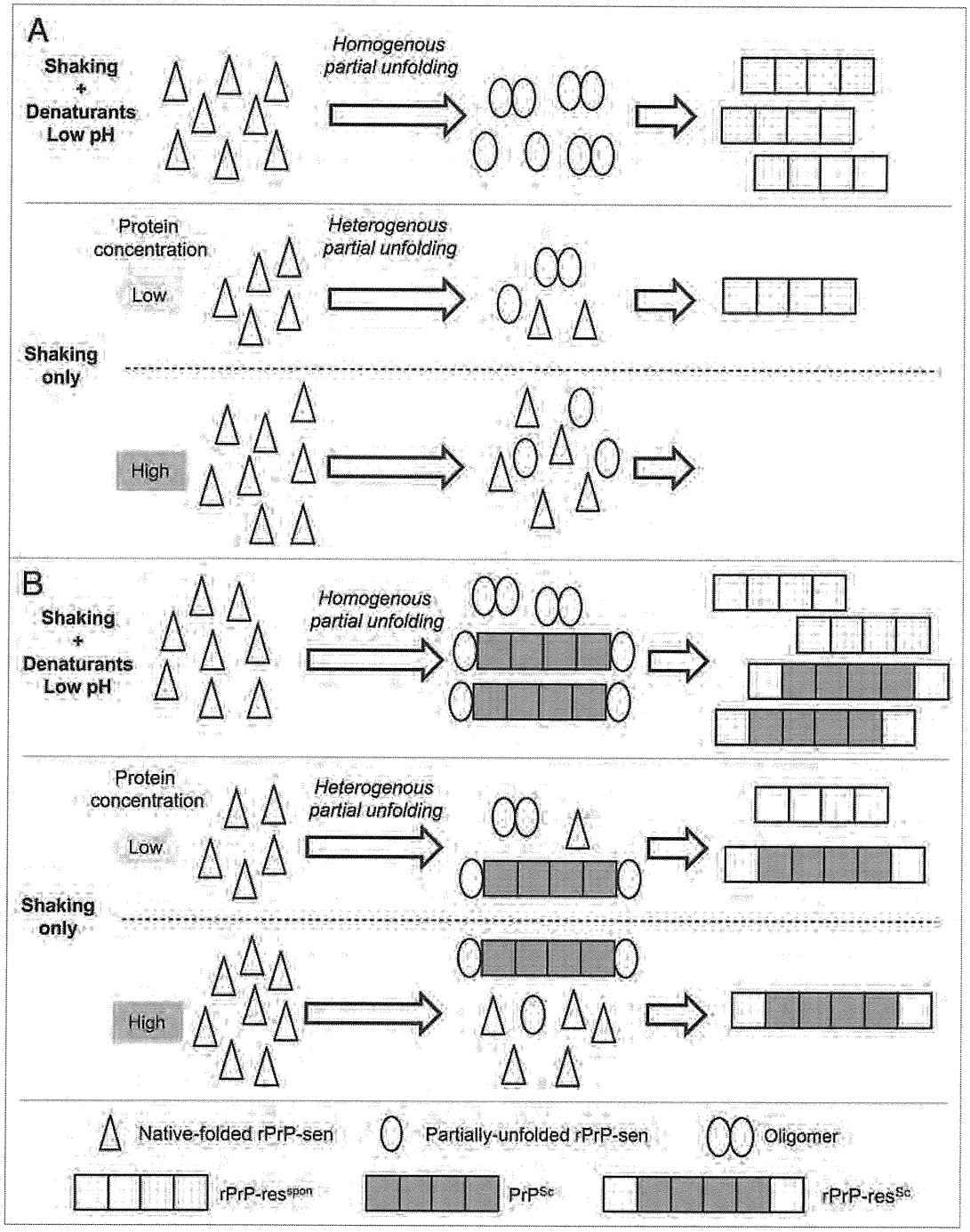

Figure 1. Hypothetical models for the inverse correlation between rPrP-sen concentration and fibril formation in a denaturant-free buffer with shaking in the absence (A) or presence of host-derived PrP^{Sc} (B). Homogeneous partial-unfolding of rPrP-sen is induced in the presence of denaturant or at low pH, leading to an increase of oligomer formation. In contrast, a heterogeneous denaturation status of rPrP-sen is presumed to be inversely proportional to the concentration in a denaturant-free buffer with shaking, resulting in a reduction of oligomer formation. It remains to be determined whether native-folded rPrP-sen can bind to PrP^{Sc} or rPrP-res polymers.

neurological disorders. Moreover, CSF is likely to contain more PrP^{Sc} and fewer impurities than blood. Examining more than 200 CSF specimens from Japanese and Australian patients, we demonstrated that RT-QUIC has greater than 80% sensitivity and absolute specificity for the detection of PrP^{Sc} in the CJD-positive CSF samples.[12] Until now, diagnostic

investigations to evaluate suspected sCJD, although of proven utility, have relied upon non-specific bio-markers, such as the detection of 14-3-3 proteins in the CSF.[25-27] The sensitivity of RT-QUIC was equivalent to and the specificity was much higher than that achieved by 14-3-3 protein measurement. Thus, the RT-QUIC provides a valuable novel means for the antemortem diagnosis of sCJD. Although most of the CSF samples we tested were 129MM, 3/4 129VV and 2/2 129MV CSF samples were positive, suggesting that RT-QUIC using 129M rHuPrP-sen as a substrate is equally valuable in all genetic subtypes of sCJD. Additionally, we recently found that RT-QUIC is potentially useful in the diagnosis of genetic human prion diseases, including Gerstmann-Straussler-Schenker disease (GSS) and fatal familial insomnia (FFI) (manuscript in preparation). While the conversion between PrP-sen and PrP^{Sc} with identical sequences is generally thought to be efficient, our findings suggest that the degree of sequence correspondence between substrate and seed can vary in the RT-QUIC reactions. In support of this concept, we observed that hamster or bovine rPrP-sen can be actively converted into rPrP-res when seeded with sCJD-PrP^{Sc}, albeit with about one log reduction in the detection limit (unpublished data). Furthermore, Orru et al. reported that the use of hamster-sheep chimera rPrP-sen provided for greater sensitivity and less spontaneous fibril formation than was observed with the homologous rHuPrP-sen in the RT-QUIC seeded with vCJD brain homogenate.[28] These results raise the possibility that rPrP-sen may also react to fibrils consisting of other proteins such as beta-amyloid, possibly resulting in a decrease in the specificity of the assay. However, we have yet to experience a single false positive in the RT-QUIC among hundreds of CSF specimens from non-CJD neurodegenerative diseases, including Alzheimer disease, we have tested. Moreover, no increase in ThT fluorescence was observed in the presence of beta-amyloid fibrils artificially formed in vitro (unpublished data). Nevertheless, further studies will be required to completely eliminate the possibility of false positives in the clinical setting. Additionally, the elucidation of the mechanism of $rPrP\text{-}res^{Sc}$ formation in the RT-QUIC, including the degree of sequence correspondence, would lead to a better understanding of the molecular basis of prion propagation.

Further Progress in RT-QUIC Technology

Recently, Caughey's group demonstrated that our RT-QUIC could be successfully applied to the detection of hamster and sheep scrapie, deer chronic wasting disease (CWD) and vCJD.[28,29] Additionally, our team has been able to detect BSE at a sensitivity equivalent to that of sCJD (manuscript in preparation). In addition, the RT-QUIC can rapidly determine the relative prion concentration when used in combination with end-point dilution analysis.[29] In another very recent study, Caughey's team showed that enrichment of PrP^{Sc} in plasma by immunoprecipitation employing the PrP aggregate-specific monoclonal IgM antibody 15B3 greatly enhances the sensitivity of RT-QUIC, especially when coupled with a substrate replacement step.[28] Together, these studies demonstrated the wide-ranging application of RT-QUIC to clinical and basic research on human and animal prion diseases.

References

1. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363-83.
2. Hamaguchi T, Noguchi-Shinohara M, Nozaki I, Nakamura Y, Sato T, Kitamoto T, et al. The risk of iatrogenic Creutzfeldt-Jakob disease through medical and surgical procedures. Neuropathology 2009; 29:625-31.
3. Ironside JW. Variant Creutzfeldt-Jakob disease. Haemophilia 5:175-80.
4. Castilla J, Saa P, Morales R, Abid K, Maundrell K, Soto C. Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Methods Enzymol 2006; 412:3-21.
5. Atarashi R. Recent advances in cell-free PrP^{Sc} amplification technique. Protein Pept Lett 2009; 16:256-9.
6. Saa P, Castilla J, Soto C. Presymptomatic detection of prions in blood. Science 2006; 313:92-4.
7. Atarashi R, Moore RA, Sim VL, Hughson AG, Dorward DW, Onwubiko HA, et al. Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat Methods 2007; 4:645-50.
8. Colby DW, Zhang Q, Wang S, Groth D, Legname G, Riesner D, et al. Prion detection by an amyloid seeding assay. Proc Natl Acad Sci USA 2007; 104:20914-9.
9. Atarashi R, Wilham JM, Christensen L, Hughson AG, Moore RA, Johnson LM, et al. Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. Nat Methods 2008; 5:211-2.
10. Orru CD, Wilham JM, Hughson AG, Raymond LD, McNally KL, Bossers A, et al. Human variant Creutzfeldt-Jakob disease and sheep scrapie PrP(res) detection using seeded conversion of recombinant prion protein. Protein Eng Des Sel 2009; 22:515-21.
11. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, et al. Cell-free formation of protease-resistant prion protein. Nature 1994; 370:471-4.
12. Atarashi R, Satoh K, Sano K, Fuse T, Yamaguchi N, Ishibashi D, et al. Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. Nat Med 2011; 17:175-8.
13. Toth SI, Smith LA, Ahmed SA. Extreme sensitivity of botulinum neurotoxin domains towards mild agitation. J Pharm Sci 2009; 98:3302-11.
14. Collins SR, Douglass A, Vale RD, Weissman JS. Mechanism of prion propagation: amyloid growth occurs by monomer addition. PLoS Biol 2004; 2:321.
15. Lansbury PT Jr, Caughey B. The chemistry of scrapie infection: implications of the 'ice 9' metaphor. Chem Biol 1995; 2:1-5.
16. Sluzky V, Tamada JA, Klibanov AM, Langer R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. Proc Natl Acad Sci USA 1991; 88:9377-81.
17. Treuheit MJ, Kosky AA, Brems DN. Inverse relationship of protein concentration and aggregation. Pharm Res 2002; 19:511-6.
18. Caughey B, Kocisko DA, Raymond GJ, Lansbury PT Jr. Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. Chem Biol 1995; 2:807-17.
19. Baskakov IV, Bocharova OV. In vitro conversion of mammalian prion protein into amyloid fibrils displays unusual features. Biochemistry 2005; 44:2339-48.
20. Jain S, Udgaonkar JB. Evidence for stepwise formation of amyloid fibrils by the mouse prion protein. J Mol Biol 2008; 382:1228-41.
21. Stohr J, Weinmann N, Wille H, Kaimann T, Nagel-Steger L, Birkmann E, et al. Mechanisms of prion protein assembly into amyloid. Proc Natl Acad Sci USA 2008; 105:2409-14.
22. Horiuchi M, Caughey B. Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state. EMBO J 1999; 18:3193-203.
23. Nishina K, Jenks S, Supattapone S. Ionic strength and transition metals control PrP^{Sc} protease resistance and conversion-inducing activity. J Biol Chem 2004; 279:40788-94.
24. Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, Windl O, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 1999; 46:224-33.
25. Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG. The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. N Engl J Med 1996; 335:924-30.
26. Zerr I, Bodemer M, Gefeller O, Otto M, Poser S, Wiltfang J, et al. Detection of 14-3-3 protein in the cerebrospinal fluid supports the diagnosis of Creutzfeldt-Jakob disease. Ann Neurol 1998; 43:32-40.
27. Satoh K, Tobiume M, Matsui Y, Mutsukura K, Nishida N, Shiga Y, et al. Establishment of a standard 14-3-3 protein assay of cerebrospinal fluid as a diagnostic tool for Creutzfeldt-Jakob disease. Lab Invest 2010; 90:1637-44.
28. Orru CD, Wilham JM, Raymond LD, Kuhn F, Schroeder B, Raeber AJ, et al. Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. MBio 2011; 2:00078-11.
29. Wilham JM, Orru CD, Bessen RA, Atarashi R, Sano K, Race B, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. PLoS Pathog 2011; 6:1001217.

Matsui et al. BMC Neurology 2011, 11:120
http://www.biomedcentral.com/1471-2377/11/120

BMC Neurology

RESEARCH ARTICLE Open Access

High sensitivity of an ELISA kit for detection of the gamma-isoform of 14-3-3 proteins: usefulness in laboratory diagnosis of human prion disease

Yuki Matsui[1†], Katsuya Satoh[2*†], Toshiaki Miyazaki[3], Susumu Shirabe[4], Ryuichiro Atarashi[2], Kazuo Mutsukura[5], Akira Satoh[5], Yasufumi Kataoka[1] and Noriyuki Nishida[2]

Abstract

Background: The gamma-isoform of the 14-3-3 protein (14-3-3 gamma) is expressed in neurons, and could be a specific marker for neuronal damage. This protein has been reported as a detectable biomarker, especially in the cerebrospinal fluid (CSF) of Creutzfeldt-Jakob disease (CJD) patients by Western blotting (WB) or enzyme-linked immunosorbent assays (ELISAs). Western blotting for 14-3-3 gamma is not sensitive, and the reported data are conflicting among publications. An ELISA specific for 14-3-3 gamma is not available.

Methods: CJD patients (n = 114 sporadic CJD patients, 7 genetic CJD, and 3 iatrogenic CJD) and 99 patients with other neurodegenerative diseases were examined in this study. The CSF samples obtained were analyzed by Western blotting for 14-3-3 gamma, and by ELISA for total tau protein. We evaluated the sensitivity and specificity of the newly developed sandwich ELISA for 14-3-3 gamma.

Results: The cut-off value of the 14-3-3 gamma ELISA was > 1, 683 AU/ml; and sensitivity was 95.2%, with 72.7% specificity. This specificity was the same for the total tau protein ELISA. Seven CJD cases were negative by WB but positive using the 14-3-3 gamma ELISA, indicating that the ELISA is more sensitive. All 21 cases of early stage CJD could be diagnosed using a combination of the 14-3-3γ ELISA and diffusion weighted MR imaging (DWI-MRI).

Conclusion: The 14-3-3 gamma ELISA was more sensitive than conventional WB, and was useful for laboratory diagnosis of CJD, similar to the ELISA for the tau protein. Using DWI-MRI and these ELISA tests on CSF, diagnosis of CJD will be possible even at early stages of the disease.

Keywords: CJD, CSF, ELISA, prion disease, 14-3-3 protein, tau protein

Background

Hshich et al.[1] reported use of the 14-3-3 protein for diagnosis of prion diseases in 1996. This protein is a reliable marker of rapid neuronal destruction, and has been detected in the cerebrospinal fluid (CSF) of several progressive neurological disorders. The 14-3-3 protein is one supportive and essential marker in the CSF of sporadic Creutzfeldt-Jakob disease (CJD) patients. Periodic sharp wave complexes (PSWC) observed on an electroencephalographic (EEG) recording and the presence of 14-3-3 in CSF are both included in the diagnostic criteria for CJD as supplied by the World Health Organization (WHO) [2]. The 14-3-3 protein is detected by Western blot (WB) in many clinical laboratories; however, conducting a WB assay to detect 14-3-3 is time consuming and expensive because the WB method consists of many steps and requires multiple investigators to discern the protein bands [3]. Thus, the development of a standard 14-3-3 protein assay and a valid criterion for quantitative assessment is urgently required.

The 14-3-3 protein has been reported to be a detectable biomarker, especially in the CSF of CJD patients, by WB or enzyme-linked immunosorbent assay (ELISA). However, the WB to detect 14-3-3 is not very sensitive

* Correspondence: satoh-prion@nagasaki-u.ac.jp
† Contributed equally
[2]Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Science, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
Full list of author information is available at the end of the article

enough and the data obtained to date differ among reports.

Previous studies have utilized an ELISA to detect 14-3-3 in the CSF [4,5] (Additional file 1, table S1), but four problems were encountered: the number of CJD patients was less than 50; the make-up of the CJD and case-control groups was not clear; the analytic means of the cut-off data for the ELISA were unclear; and the specificity was very low. We attempted to improve upon these four problems and attempted to develop a new, specific, 14-3-3γ ELISA.

We developed a specific ELISA using the gamma isoform of 14-3-3 (14-3-3γ). Additionally, results from the WB and ELISA analyses were compared. We analyzed 124 human prion disease cases and 99 human non-prion disease cases using the 14-3-3γ ELISA and assessed if quantification of 14-3-3 might be helpful in their differentiation.

Methods

1. Patients

Patients with suspected CJD were recruited from hospitals all over Japan for the purpose of conducting biochemical CSF assays. From more than 300 requests, a follow-up study was performed with 124 CJD patients. The cases were classified as sporadic CJD (n = 114), genetic CJD (n = 7; four cases with a V180I mutation, two cases with an M232R mutation and a single case with an E200K mutation in the prion protein gene), or iatrogenic CJD (dura-associated CJD; n = 3). A total of 114 sporadic CJD patients and 3 iatrogenic CJD patients did not express a *prion protein gene* (*PRNP*) mutation. All 124 cases contained MM at codon 129 and EE at codon 219 of the *PRNP*.

Subjects for the present study consisted of 114 sporadic CJD patients with confirmed CJD diagnosis. All 114 sporadic patients fulfilled the WHO diagnostic criteria for CJD. All sporadic CJD cases were typical with respect to clinical findings, clinical time course, neuroimaging [FLAIR and diffusion-weighted magnetic resonance imaging (DWI-MRI) with high signal abnormalities in the caudate nucleus and putamen, or at least two cortical regions] and PSWCs via EEG.

A total of seven sporadic CJD cases (four males, three females) were definite, and 107 sporadic cases were probable among the 114 sporadic CJD patients. Two cases (V180I; females) were definite cases among the seven genetic CJD cases, and one case was a definite among the three iatrogenic CJD cases.

For control samples, CSF was collected from 99 patients who suffered from one of the following disorders: dementia of Alzheimer's type (DAT; n = 54, 33 male, 21 female), cerebrovascular dementia (n = 7, 5 male, 2 female), Parkinson's disease (n = 5, 4 male, 1 female), progressive supranuclear palsy (PSP; n = 3, 2 male, 1 female), frontotemporal lobular degeneration (n = 2, 1 male, 1 female), Huntington's disease (n = 1; 1 male), corticobasal degeneration (n = 2, both female), amyotrophic lateral sclerosis (n = 3, 1 male, 2 female), limbic encephalitis (n = 2, 1 male, 1 female), mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS; n = 4, 2 male, 2 female), paraneoplastic cerebellar disorder/Lambert-Eaton myasthenic syndrome (PCD/LEMS; n = 2, 1 male, 1 female), temporal epilepsy (n = 4, 1 male, 3 female), mild cognitive impairment (n = 3, 1 male, 2 female), and dementia etiology unknown (n = 3, 1 male, 2 female). Additionally, CSF was obtained from healthy volunteers (n = 4, 2 male, 2 female).

All subjects were examined and CSF samples collected, divided into aliquots, and stored at -80°C until required. All assays were performed simultaneously to avoid repeated freezing and thawing of samples. The CSF samples were used within 1 month of collection.

We previously reported about 21 patients that suffered from early-stage CJD, defined as cases in the 6 weeks following the onset of the disease [6]. We used the CSF samples and clinical findings of these 21 patients. This study was approved by the Medical Ethics Committee of Nagasaki University, School of Medicine (06012755; UMIN000003301), and the participants provided written informed consent.

2. Analysis of CSF by WB for 14-3-3

The CSF samples were collected, aliquoted, and stored at -80°C until required. All assays were performed simultaneously to avoid repeated freezing and thawing of samples. Immunoassays for 14-3-3 protein were performed as previously described [3]. Polyclonal antibodies specific for 14-3-3γ were obtained from Immuno-Biological Laboratories (18647; Gunma, Japan) and were used at a dilution of 1:500. Polyclonal antibodies specific for all isoforms of 14-3-3 were obtained from Santa Cruz Biotechnology (sc-1657; Santa Cruz, CA, USA) and used at a dilution of 1:1, 000. All samples were analyzed using the same antibody to ensure comparable sensitivities. Protein detection was performed using an enhanced chemiluminescence detection kit (Amersham Buchler Company). Detection of 14-3-3 in the CSF samples was performed as previously described [3]. All assays were performed by two independent researchers.

3. Analysis of total tau protein in CSF samples

Detection of total tau protein in the CSF samples was performed as previously described [6].

4. ELISA detection of 14-3-3 in CSF

We immunized rabbits and mice with eight 14-3-3γ peptides and obtained three monoclonal antibodies (clones #4-#6; additional files 2, table S2) and three polyclonal antibodies (clones #1-#3; additional file 2, table S2). These six antibodies were characterized and the best

combination of antibodies for the sandwich ELISA was determined (clones #1 and # 6; Additional file 3, figure S1). The sandwich ELISA for detection of 14-3-3γ was used to analyze CSF samples from 124 CJD patients and the remaining 99 patients with other diseases. The ELISA assay was sensitive enough to detect 14-3-3γ in the range 125-16, 000 AU/ml in human CSF samples. The ELISA plates were coated with antibody and then 50 μl of CSF and 50 μl of sample dilution buffer was added to each well. The samples were incubated for 1 hour at room temperature. Following incubation in the appropriate secondary antibody, samples were reacted with horseradish peroxidase and the optical density of each well at 450 nm was determined using a microplate reader. All processes were completed within a 4-hour period.

5. Analysis by the real-time QUIC (RT-QUIC) method

Analysis by the RT-QUIC method was performed as previously described [7].

6. Statistical analysis

SPSS version 11.0 software was used to perform all statistical analyses. Standard measures of diagnostic test validity were used to identify true-positive, true-negative, false-positive, and false-negative results. The levels of 14-3-3γ in the 124 CJD patients and remaining 99 patients with other diseases were used for these calculations [receiver operating characteristic (ROC) analysis].

7. MRI Protocol and PSWC on EEGs

We have described the MRI protocol and PSWC on EEGs previously [6].

Results

1. Detection of 14-3-3 by WB in CSF samples

In the CJD group, 14-3-3 was detected in all CSF samples (n = 124; Tables 1 and 2). The 14-3-3 protein was detected in two non-CJD patients with DAT, one with CVD, two with Wernicke's encephalopathy, and three patients with limbic encephalitis. The WB sensitivity and specificity for 14-3-3γ in CJD patients was 87.1 and 84.8%, respectively (Tables 1 and 2, Figure 1-a, b, c). In other hand, WB sensitivity and specificity for all isoforms of 14-3-3 in CJD patients was 91.4 and 78.8% (Tables 1 and 2).

Table 1 The sensitivity and the specificity of ELISA of 14-3-3 γ protein, the detection of 14-3-3 γ protein and 14-3-3a protein in WB methods

	14-3-3 γ-isoform		14-3-3 all isoforms
	ELISA	WB	WB
sensitivity (%)	95.2	87.1	91.4
specificity (%)	72.7	84.8	78.8

2. Detection of total tau protein in CSF Samples

The levels of total tau protein in CSF were determined in 223 patients, and significant differences were observed among individuals (Tables 1 and 2). The level of total tau protein was highest in the CJD group, ranging from 1, 048-146, 087 pg/ml (mean ± SD, 7, 174 ± 6, 558 pg/ml). The CJD patients expressed higher levels of total tau proteins compared with patients suffering from other neurological disorders patient ($p < 0.01$; Tables 1 and 2, Figure 1).

3. Detection of 14-3-3 in CSF samples by ELISA

The levels of 14-3-3 in CSF were determined in 223 patients, with significant differences observed among individuals. The level of 14-3-3 was greatest in the CJD group, ranging from 135-75, 373 AU/ml (mean ± SD, 263, 549 ± 21, 525 AU/ml; Table 2-a). In the DAT group, the concentration of 14-3-3 was between 0-2, 410 AU/ml (mean ± SD, 1, 537 ± 751.2 AU/ml). The CVD group exhibited levels similar to those in the DAT group, with a range of 521-1, 512 AU/ml (mean ± SD, 975.9 ± 332.4 AU/ml). The level of 14-3-3 in CJD patients was also higher compared with patients suffering other neurological disorders, including the CVD and DAT groups ($p < 0.01$). A cut-off (1, 683 AU/ml) was determined by an ROC curve (Figure 1-a). The sensitivity and specificity for detection of 14-3-3 in CJD patients was 95.2 and 72.7%, respectively (Tables 1 and 2, Figure 1-a, b, c). We were able to demonstrate that these ELISA results were reproducible (additional file 4, figure S2-a, b)

4. Analysis of biochemical markers in 21 early-stage CJD patients

We previously published a report outlining 21 subjects that suffered from early-stage CJD, which was defined as cases in the 6 weeks following the onset of disease [6]. The sensitivities for detection of total tau protein and 14-3-3 in CSF were analyzed to determine their usefulness as diagnostic markers of early-stage CJD. Total tau and 14-3-3 proteins were detected in 95.2 and 76.2%, respectively, of the early-stage CJD patients. In all 21 patients, the data exceeded the cut-off data (> 1, 683 AU/ml) for the 14-3-3 ELISA (Table 3). Additionally, DWI-MRI was used to positively identify 90.5% of these cases. All cases were able to be diagnosed using a combination of DWI-MRI and 14-3-3γ ELISA.

5. Analysis of CJD patients where 14-3-3γ was not detected by WB yet the concentration of 14-3-3γ exceeded the ELISA cut-off level (Table 4)

In seven of the 124 CJD patients, 14-3-3γ was not detected by the WB but their values were above the cut-off level (1, 683 AU/ml) as determined by the 14-3-3γ

Table 2 Analysis of Western blots method and ELISA of 14-3-3 protein of CSF in 124 CJD patients and 99 patients with other neurological disorders and rapid progressive dementia

disease	number	male	female	14-3-3 γ					14-3-3 all isoforms
				ELISA				WB	WB
				average	SD	min	max		
CJD	124	69	55	263, 549	21, 525	135	75, 373	108/124	114/124
DAT	54	33	21	1, 537	751.2	0	2, 410	3/54	7/54
CVD	7	5	2	975.9	332.4	521	1, 51 2	0/7	2/7
PD	5	4	1	542.2	20.3	521	565	0/5	0/5
PSP	3	2	1	223.3	226.6	38	476	0/3	0/3
FTLD	2	1	1	425	236.2	258	592	0/2	0/2
HD	1	1	0	556	0.	556	556	0/1	0/1
CBD	2	2	0	449.5	245.4	276	623	0/2	0/2
ALS	3	1	2	303.7	184.8	179	516	0/3	0/3
limbic encephalitis	2	1	1	4440.5	1935.4	3, 072	5, 809	2/2	2/2
MELAS	4	2	2	5, 069.5	222.7	4, 912	5, 227	4/4	4/4
PCD/LEMS	2	1	1	4, 387	217.8	4, 233	4, 541	2/2	2/2
temporal epilepsy	4	1	3	3, 356.8	1332.4	1, 530	4, 696	4/4	4/4
MCI	3	1	2	603	20.8	591	627	0/3	0/3
Dementia, etiology unknown	3	1	2	410.3	150.5	258	559	0/3	0/3
healthy subject	4	2	2	0	0	0	0	0/4	0/4

CJD: Creutzfeldt-Jakob disease, DAT: Dementia of Alzheimer's type, CVD: Cerebral Vascular Disorder, PD: Parkinson's disease, PSP: progressive supranuclear palsy, FTLD: frontotemporal lobular degeneration HD: Huntington's disease, CBD: corticobasal degeneration, ALS: amyotrophic lateral sclerosis, MELAS: Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, Stroke-like episodes, PCD: paraneoplastic cerebellar degeneration, LEMS: Lambert-Eaton myasthenic syndrome, MCI: mild cognitive impairment,

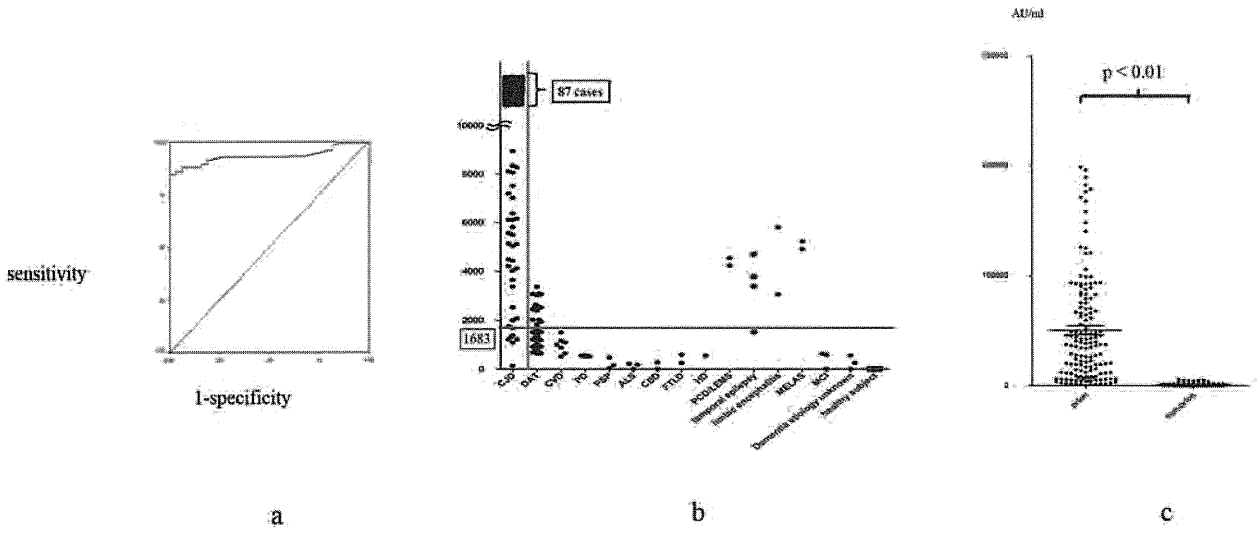

Figure 1 ELISA analysis of 14-3-3 protein in CSF from patients with CJD and other neurological disorders. 1-a. Receiver operating curve characteristics at different cut-off points for the 14-3-3 ELISA applied to CSF samples. 1-b. Results of the 14-3-3 ELISA analysis in CSF from patients with CJD and other forms of dementia. CJD, Creutzfeldt-Jakob disease; DAT, dementia of Alzheimer's type; CVD, cerebrovascular disorders; PD, Parkinson's disease; PSP, progressive supranuclear palsy; FTLD, frontotemporal lobular degeneration; HD, Huntington's disease; CBD, corticobasal degeneration; PCD/LEMS, paraneoplastic cerebellar disorder/Lambert-Eaton myasthenic syndrome; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MCI, mild cognitive impairment. 1-c. Comparison of CJD patients and non-prion patients.

Table 3 Summary of the detection of ELISA of 14-3-3 protein, the detection of WB method of 14-3-3 protein and in CSF for 21 patients with early-stage CJD

Age	Sex	CJD type	d.w.	CSF				MRI
				ELISA of 14-3-3 protein AU/ml	the detection of WB method of 14-3-3a protein	the detection of WB method of 14-3-3γ protein	Real-time QUIC	DWI
71	m	sp probable	0	4, 157	-	-	+	+
77	m	sp probable	2	10, 812	+	+	+	+
64	f	sp probable	4	11, 812	+	+	+	+
73	m	sp probable	4	3, 850	+	+	-	+
67	m	sp probable	4	10, 814	+	+	+	+
76	m	sp probable	4	6, 772	+	+	+	+
80	f	sp probable	4	9, 850	+	+	+	+
63	f	sp probable	4	2, 987	-	-	-	+
67	m	sp probable	4	3, 553	+	+	+	+
70	f	sp probable	4	3, 729	-	-	-	+
63	m	sp probable	4	6, 133	+	+	+	+
63	m	sp probable	4	8, 297	+	+	+	+
67	f	sp probable	5	3, 372	+	-	-	-
74	m	sp probable	5	7, 184	+	+	+	+
69	f	sp definite	6	5, 600	+	+	+	+
54	f	sp definite	6	1, 897	-	-	-	+
70	f	ia probable	6	9, 460	+	+	+	-
70	f	fa probable	6	4, 417	+	+	+	+
64	f	sp probable	6	3, 888	+	+	+	+
51	f	ia probable	6	6, 313	+	+	+	+
74	f	sp probable	6	6, 133	+	+	+	+

sp = Sporadic CJD; ia = iatrogenic CJD; fa = familiar CJD; d.l. = diagnostic level based on the WHO and the Masters criteria; d.w. = duration from the onset of the disease to the diagnostic examination. In all 21 cases, codon 129 of PRNP was Met/Met homozygous, whereas codon 219 was Glu/Glu homozygous. Total protein contents of all the patients stayed within the normal range. All patients in this study were Asian.

ELISA (Table 4). The clinical courses of disease for the five sporadic CJD patients were longer when compared with the classical CJD patients.

6. Analysis of CJD patients where 14-3-3γ was not detected using WB and 14-3-3γ concentration was below the cut-off level for the ELISA (Table 5)

For seven of the 124 CJD subjects, 14-3-3γ was not detected using the WB method and the concentration of the protein was below the cut-off level (1, 683 AU/ml) as determined by the 14-3-3γ ELISA (Table 5). Five of the genetic CJD patients had the V180I mutation, and the remainder had the M232R slow-type mutation. The clinical courses of the disease in the five genetic CJD patients were longer than for the classical CJD patients.

7. Analysis of non-CJD patients with positive 14-3-3γ ELISA diagnoses (Table 6)

The results from 23/99 of the non-CJD patients demonstrated that the concentration of 14-3-3γ exceeded the cut-off limit. When the concentration of 14-3-3γ was

Table 4 Analysis of CJD patient s that were not detected in 14-3-3γ protein of WB methods but were beyond the cut-off level (1, 683 AU/ml) in 14-3-3 protein ELISA

Age	Sex	type	Polymorphism of codon129 in PRNP gene	Mutation of PRNP gene	ELISA of 14-3-3 protein (AU/ml)	Real-time QUIC
69	male	sp	MM	-	2, 101	+
33	female	sp	MM	-	1, 852	+
70	female	sp	MM	-	2, 452	-
73	female	sp	MM	-	2, 531	-
64	male	sp	MM	-	5, 493	+
65	male	fa	MM	E200K	4, 621	+
79	female	sp	VV	-	2, 342	+

sp: sporadic type, ge: genetic type, MM: methioine homozygotes, VV: valine homozygotes, +: positive, -: negative

Table 5 Analysis of CJD patient s that were not detected in 14-3-3γ protein of WB methods and were below the cut-off level (1, 683 AU/ml) in 14-3-3 protein ELISA

Age	Sex	type	Polymorphism of codon129 in PRNP gene	Mutation of PRNP gene	ELISA of 14-3-3 protein (AU/ml)	Real-time QUIC
85	female	ge	MM	V180I	1, 086	-
84	female	ge	MM	V180I	1, 222	-
84	male	ge	MM	V180I	1, 313	-
84	female	ge	MM	V180I	1, 383	-
75	male	ge	MM	M232R	1, 676	+
78	male	sp	MM	-	135	-
66	male	sp	MM	-	1, 224	+

sp: sporadic type, ge: genetic type, MM: methioine homozygotes, VV: valine homozygotes, +: positive, -: negative

greater than 3, 000 AU/ml, the protein in the samples could also be detected by WB. Patients diagnosed with PCD/LEMS, MELAS, limbic encephalitis, temporal epilepsy, and a part of DAT were pseudo-positive, but negative using the RT-QUIC method.

Discussion

We attempted to develop a sandwich ELISA using combinations of the specific γ-isoform antibodies (clones #1, #2 and #6: additional file 2, table S2), but only one combination of antibodies (#1 and #6: additional file 2, table S2) showed a dose-dependent reaction (Additional files 3, figure S1-a). The #1 and #6 antibodies reacted with the γ-isoform of 14-3-3.

There are clear advantages for using an ELISA over a WB for the detection of 14-3-3 in CSF samples. The WB method requires many steps for the detection of the target protein, with the WB protocol varying between laboratories. Thus, it is very difficult to obtain consistent data regarding the number, location and intensity of banding patterns. Moreover, the WB method can assay several samples simultaneously. It is an unsuitable method for patients in urgent need of treatment. However, the ELISA method allows for simultaneous processing of numerous CSF samples and appears to be the best method for use in a clinical setting.

In this study, we developed a sensitive and precise ELISA method for the quantification of 14-3-3 in CSF. The ELISA analysis of CSF in CJD patients was stratified by diagnosis category, and indicated a significantly higher 14-3-3 level in definite as well as probable CJD patients, compared to the CSF from patients with other neurological disorders.

Additionally, seven cases were analyzed by ELISA as the protein levels could not be determined by WB. These seven cases were classified into two categories (Tables 5 and 6). The first category consisted of samples with a total tau protein concentration exceeding 1, 300 pg/ml, with the concentration of 14-3-3 greater than 1, 683 AU/ml as determined by the ELISA. The second category consisted of samples with a total tau protein concentration less than 1, 300 pg/ml, but 14-3-3 concentration greater than 1, 683 AU/ml according to the ELISA (Table 5). All samples from CJD patients were assayed using the WB method for detecting 14-3-3 and the ELISA method for detecting total tau protein. This is because four patients exhibited a positive reaction for total tau (> 1, 300 pg/ml) and a negative reaction for 14-3-3 protein by WB; these four patients were also positive using the 14-3-3 ELISA (Table 4 and 5). We suggest that it might be unnecessary to check for both 14-3-3 and total tau proteins.

The present study varied from previous ELISA studies. In previous studies, all isoforms of 14-3-3 were detected, whereas in the present study we only detected the γ-isoform. The amount of 14-3-3γ was elevated in the CSF of CJD patients, consistent with previous WB studies. In a particular study [5], an ELISA method that measured all isoforms of 14-3-3 proved to be very useful. However, this system is not commercially available. Additionally, data from the ELISA method cannot be compared to previous results based on the β- or γ-isoform. However, the method described in the present study allows for these comparisons, with high specificity and sensitivity compared to other studies [2]. The differences in sensitivity and specificity for 14-3-3 diagnosis in various studies are likely due to the lack of uniformity between the diseases, as well as the number of diseases associated with 14-3-3. Previous studies [8,9] have shown that WB sensitivity and specificity for 14-3-3 are useful for diagnosis. However, the ELISA method is standardized, allowing for data comparison between subsequent studies. All ELISA procedures are completed within 4 hours, but the WB method requires 2-3 days. In addition, the ELISA assay can simultaneously analyze 40 samples from eight individuals.

Our previous report identified that the detection of total tau protein combined with DWI-MRI identified 98% of the early-stage cases. Pennington C et al [10]