A chicken monoclonal antibody with specificity for the N-terminal of human prion protein

Haruo Matsuda a *, Hiroyuki Mitsuda a, Naoto Nakamura a, Shuichi Furusawa a, Shirou Mohri b, Tetsuyuki Kitamoto c

a Department of Immunobiology, Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan
b Center of Biomedical Research, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan
c Department of Neurological Science, Tohoku University School of Medicine, 2-1 Seiryou-cho, Aoba-ku, Sendai 980-8575, Japan

Received 30 June 1998; received in revised form 22 September 1998; accepted 26 October 1998

Abstract

Chickens were immunized with human prion protein (PrP) peptide H25 (amino acid residues 25–49) coupled to keyhole limpet hemocyanin. From a fusion experiment using the chicken fusion partner cell line MuH1 and immune spleen cells, one mAb, HUC2-13, was generated which reacted with the peptide. HUC2-13 was specific for a pentapeptide (RPKPG) of the N-terminal of the peptide H25. In Western blotting analysis, the mAb reacted with PrP materials from a human Creutzfeldt–Jakob disease (CJD) case and the membrane fraction from normal murine brain, but not with the same materials pretreated with proteinase K. When compared with the HUC2-13 and the conventional mouse mAb 3F4, the background stainings using the HUC2-13 were minimal. In immunohistochemistry, the HUC2-13 stained positively with kuru plaques in brain sections from patients with Gerstmann–Straussler syndrome (GSS), and also reacted with synaptic structures of the CJD patients. However, any immunolabelings using the HUC2-13 were not observed in the section from a patient with amyotrophic lateral sclerosis (ALS) as CJD-negative control. These results indicate that the mAb HUC2-13 is a suitable tool for immunological and diagnostic analyses of prion disease in humans and other mammals.

Keywords: Chicken monoclonal antibody; Prion protein; Creutzfeldt–Jakob disease; Gerstmann–Straussler syndrome

1. Introduction

Scrapie is a naturally occurring transmissible neurodegenerative disease in sheep and goats and is the prototype of all transmissible spongiform encephalopathies (TSE) which are characterized by accumulation of an abnormal form of a cellular prion protein (PrPSc) [1,2]. The abnormal form of PrP is partially...
proteinase resistant (PrPsc) whereas PrPc can be completely degraded by proteinase K.

The diagnosis of TSE is usually based on clinical symptoms and histopathology. The PrPsc is also useful as a diagnostic marker, using specific polyclonal and monoclonal antibodies (mAbs) raised in mammals [3–14]. However, the high homology (> 95%) between mammalian PrP amino acid sequences [15–17] indicates that there are few specific epitopes. Numerous PrP-specific rabbit polyclonal antibodies as well as a limited number of mouse mAbs have been raised [3–13]. The lower homology (< 40%) between avian and mammalian PrP suggested that birds might be useful for the generation of specific antibodies to mammalian PrP. Recently, a panel of chicken antisera against synthetic sheep PrP peptides was described [14]. More recently, we generated a total of 19 chicken mAbs against synthetic bovine PrP peptide [18] which displayed different reactivities to bovine, human, murine, ovine and hamster PrP amino acid sequences.

Rabbit antiserum anti-PrP-N which was raised against a peptide residues 25–49 of human PrP [19] reacted specifically with kuru plaques of brain tissues from a patient with Gerstmann–Straussler syndrome (GSS). The anti-PrP-N immunostained the periphery, but not the center of large amyloid plaques. In this study, we attempted to generate chicken mAbs against the human N-terminal PrP peptide, provisionally named H25. We describe here some properties of the chicken mAb HUC2-13.

2. Materials and methods

2.1. Cell line

The chicken B-cell line, MuH1 [20], which is deficient in thymidine kinase activity and resistant to ouabain, was used as fusion partner cells. The cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 38.5°C.

2.2. Synthetic PrP peptides

Human synthetic PrP peptide H25 (amino acid residues 25–49; Fig. 1) was used as antigen. For immunization, an additional cysteine residue was added at the C-terminal to conjugate the peptide to the carrier protein keyhole limpet hemocyanin (KLH) by use of m-maleimidobenzoic acid–N-hydroxysuccinimide ester (Pierce, USA) according to the manufacturers instructions.

2.3. Immunization

Three 1-month-old H-B15 inbred chickens were used. The chickens were immunized intramuscularly with 100 µg H25-KLH in 0.5 ml phosphate-buffered saline (PBS) together with an equal volume of alum solution. Four, 7 and 10 weeks later, they received the same antigen without alum by the intravenous route.

2.4. Cell fusion

The MuH1 cells were hybridized with the immune spleen cells 3 days after the final injection of antigen by the method described previously [20]. Cell fusion was carried out at a MuH1/spleen cell ratio of 1:3. Hybridomas producing appropriate antibodies were cloned in soft agar.

2.5. Determination of chicken mAb

Specificity of chicken sera and mAbs was initially determined by an ELISA using H25 peptide. ELISA plates were sensitized with 50 µl per well of H25 peptide at a concentration of 10 µg ml–1 in PBS and were incubated overnight at 4°C. The plates were then blocked with 200 µl per well of 25% of BlockAce (Yukizirushi, Japan) for 1 h at room temperature, and rinsed once with PBS containing 0.05% Tween 20 (PBTS). After a further 1-h incubation with 50 µl of culture supernatant, the plates were washed with PBTS, and then incubated with alkaline phosphatase-conjugated goat anti-chicken IgG (H+L) (Kirkegaard and Perry Laboratories, USA). The reaction was visualized by the addition of p-nitrophenyl phosphate (1 mg ml–1) in 0.1 M glycine buffer containing 1 mM MgCl2 and 1 mM ZnCl2, pH 10.4. Optical density measurements of each well were made at 405 nm using a Microplate Reader MPR A4 (TOSOH, Japan).

Immunoglobulin class of positive mAb
was also performed by ELISA using as secondary antibodies mouse mAbs specific for chicken IgG heavy chain (1γ, IgG1) or IgM heavy chain (4μ, IgM) [20], peroxidase-conjugated goat anti-mouse Ig (G+M) (Kirkegaard and Perry Laboratories, USA) and substrate solution.

The IgG contents secreted in culture supernatants from hybridomas were determined by ELISA technique. Briefly, ELISA microplates were coated with mouse mAb specific for chicken IgG heavy chain (1γ) (50 μl diluted 1/500) at 4°C overnight. Plates were post-coated with 200 μl per well of BlockAce for 1 h at room temperature and rinsed once with PBST. Diluted culture supernatant (1/20) was added to each well and incubated for 1 h at 37°C in the plates. After washing, 50 μl of peroxidase-conjugated rabbit anti-chicken IgG (Kirkegaard and Perry Laboratories, MD) diluted 1/2000 with PBS were added and incubated for 1 h at 37°C. Color development and measurement were done as described before. The concentrations of IgG were calculated from their OD values. The suitability of these determinations was confirmed using the known diluted standards of IgG.

2.6. Epitope mapping

Twenty one overlapping pentapeptide sequences with a shift of one amino acid residue were generated to scan the 25 amino acid residue H25 peptide sequence. The synthesis was performed on a cellulose membrane sheet by using Auto-Spot peptide Synthesizer ASP 222 (ABIMED, Germany). For identification of epitope sequence of mAbs generated in this study, peroxidase-conjugated goat anti-chicken Ig (G+M) (Kirkegaard and Perry Laboratories, USA), diaminobetidine and H2O2 were used.

2.7. Tissue preparation and Western blotting

To detect PrPsc, we used cerebral tissue (360 mg) from a patient with sporadic CJD. Tissue was homogenized with 2 ml of 40 mM Tris-HCl pH 7.5/10 mM NaCl/6 mM MgCl2/700 U DNase 1 (Takara, Japan). The homogenate was incubated at 25°C for 30 min. The tissue was then again homogenized in 1 ml of 30% Sarkosyl. The homogenate was centrifuged at 22 000 x g for 30 min. Proteinase K (25 μg) was added to the supernatant (S22) and the mixture was incubated at 37°C for 60 min. In another preparation, we omitted this proteinase digestion. With or without proteinase digestion, S22 was centrifuged at 400 000 x g for 60 min. The resulting pellet was boiled with Laemmli’s sample buffer (100 μl (100 mg original wet tissue weight)^−1). For comparison with the chicken mAb obtained in this study and the conventional PrP-specific mouse mAb 3F4 [10], 5 μl of the 10% homogenate of the brain tissue from a patient with CJD were also used applied on the 15% SDS-polyacrylamide gel.

To detect PrPc, we prepared the membrane fraction of brain of NZW mouse without CJD infection. Tissue (200 mg) was homogenized in 2 ml of 10 mM Tris-HCl/100 mM NaCl/proteinase inhibitor cocktail (Behring, Germany). The homogenate was centrifuged at 800 x g for 5 min. The supernatant (S8) was centrifuged again at 100 000 x g for 60 min. The resulting pellet was boiled in 1% SDS solution, and the protein concentration was measured by the BCA kit (Pierce, USA).

For Western blotting, we ran PrPsc samples corresponding to 10 mg original wet weight through 13.5% polyacrylamide gel, and the PrPc samples (50 μg protein content per lane) in 15% polyacrylamide gel. The procedures of membrane transfer and immunostaining were described elsewhere [21]. Non-diluted culture supernatant of chicken hybridoma cells was used as the first antibody. The conventional mouse mAb 3F4 was also used as a positive antibody. For the second antibody, we used alkaline phosphatase-conjugated affinity-purified goat anti-chicken IgG (Kirkegaard and Perry Laboratories, USA) in the chicken antibody system and alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, USA) in the mouse antibody system.

2.8. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections from patients with CJD, GSS or amyotrophic lateral sclerosis (ALS) were used. To enhance the immunostaining, the tissue sections were treated by the hydrolytic autoclave procedure. This procedure and the following immunostaining steps were described elsewhere [21]. Non-diluted culture supernatant of chicken hybridoma cells was used as the first antibody.
For the second antibody, we used the biotinylated affinity-purified goat anti-chicken IgG (Kirkegaard and Perry Laboratories, USA).

3. Results

3.1. Generation of chicken mAb against H25 peptide

All chickens immunized with H25-KLH produced specific antibody to the H25 peptide. The MuH1 cells were hybridized with the immune spleen cells from one of them 3 days after the final injection of antigen by the method described previously [20]. Finally, one cloned hybridoma producing antibodies against the H25 peptide was identified. The mAb, named as HUC2-13, obtained in this study was classified as IgG (IgY). The IgG content of their culture supernatant was 3.5 \( \mu g \) ml\(^{-1}\) when the content was determined in their culture condition at the cell concentration of \( 10^6 \) cells ml\(^{-1}\) in growth medium for 3 days.

In the epitope mapping for HUC2-13, the mAb reacted only with one pentapeptide (RPKPG) from the N-terminal of H25 peptide and did not react with the other 20 pentapeptides (Fig. 2).

3.2. Western blotting and immunohistochemistry for HUC2-13

To determine further specificity of the mAb HUC2-13, Western blot and immunohistochemical analyses were performed. In these experiments, the non-diluted supernatant of the hybridoma culture was used as the first antibody.

In Western blots, the mAb HUC2-13 reacted with 10% homogenate of the brain tissue from a patient with CJD as well as the conventional mouse mAb 3F4 (Fig. 3a). The mAb HUC2-13 also reacted with PrP\(^{sc}\) omitting proteinase K digestion, but did not react with proteinase K-digested PrP\(^{sc}\) (PrP 27-30) (Fig. 3b, lanes 1 and 2), although the mAb 3F4 reacted with PrP\(^{sc}\) treated with or without proteinase K (Fig. 3b, lanes 3 and 4). The HUC2-13 also recognized PrP\(^{sc}\) from the membrane fraction of mouse brain (data not shown). In these Western blot analyses, the background stainings using the mAb
HUC2-13 were minimal when compared with HUC2-13 and 3F4 (Fig. 3).

In immunohistochemical analysis, the HUC2-13 stained the kuru plaques of the GSS patient (Fig. 4). The periphery, but not the centers, of the spherical kuru plaques was mainly stained. The mAb also stained the diffuse synaptic-type PrP accumulations of the CJD patients (Fig. 5a). The diffuse stainings were recognized in the molecular layer and the granular cell layer of cerebellar cortex. However, any immunolabelings were not observed in the section from cerebellar cortex of a patient with ALS as CJD-negative control (Fig. 5b).

4. Discussion

Specific antibodies against mammalian PrP have been raised mainly in rabbits [3–8]. Mouse mAbs directed to PrP [6,9,10] are fewer due to higher homology (>95%) of PrP between mouse and other mammalian species. Recently, there have been a few reports of PrP-specific mouse mAbs [11–13]. Krasemann et al. produced mAbs by immunizing PrP0/0 mice with DNA or RNA coding for human PrP [11]. Phage display mouse mAbs directed to PrP have also been raised using mice in which the PrP gene was ablated (Prnp0/0) [12]. More recently, mouse mAb directed to PrPsc was generated by using PrP-null mice immunized with recombinant bovine PrP [13].

With the chicken mAb system, we expected the production of specific antibodies against mammalian PrP without the use of PrP-null animals. Previously, we developed a total of 19 chicken mAbs reactive to bovine PrP peptide B204 (amino acid residues 204–220) from one fusion experiment [18]. These mAbs to B204 were divided into five groups based on their species reactivities. The results indicated that the generation of chicken mAbs appears to be more potentially successful than that of mAbs using the PrP-null mice. Recently, many kinds of chicken antisera to synthetic ovine PrP peptides were raised [14]. However, most of these antisera were cross-reactive among different species of mammalian PrPs. The HUC2-13 reported here was also cross-reactive antibody, but it was due to the use of a highly conserved peptide, H25 (Fig. 1), of mammalian PrP. The chicken mAb seems more producible and valuable than chicken antisera on generation of species-specific antibody to mammalian PrP and on the applications.

Fig. 4. Immunostaining of the cerebral cortex from a patient with GSS. The mAb reacted with the kuru plaques. The section was counterstained with hematoxylin. ×213.

Fig. 5. Immunohistochemistry with HUC2-13. (a) Cerebellar cortex of a patient with CJD. Diffuse stainings were recognized in the molecular layer (Mol) and the granular cell layer (Gr). (b) Cerebellar cortex of a patient with ALS. The sections were counterstained with hematoxylin. ×80.
The amino acid sequence of the H25 peptide is shared by human, mouse and hamster, while the same region in bovine, ovine and rabbit includes one additional amino acid (Gly) (Fig. 1). Among PrP residues 25–51, only 9 or 10 are conserved between chickens and mammals (Fig. 1). It should, therefore, be possible to generate mAbs against interspecies-specific epitopes. However, the HUC2-13 obtained here reacted with a pentapeptide (RPKPG) within the N-terminal of the H25 peptide conserved among human, mouse, hamster, bovine, ovine and rabbit PrPs (Figs. 1 and 2).

The specificity of HUC2-13 was also confirmed by Western blotting and immunohistochemical analyses. In Western blotting analysis, the background stainings using the HUC2-13 were minimal when compared with HUC2-13 and the conventional mouse mAb 3F4 specific for PrP [10] (Fig. 3). In immunohistochemical analysis, the mAb HUC2-13 labeled the kuru plaques of the GSS patient with the same staining pattern obtained with rabbit anti-PrP-N [20] and the diffuse synaptic-type PrP accumulations of the CJD patient, while giving minimal background stainings (Figs. 4 and 5a). In the immunohistochemical study, the brain section from a patient with ALS used as CJD-negative control was not immunolabeled with the HUC2-13 (Fig. 5b).

In conclusion, our results indicate that the HUC2-13 is useful as a reagent for detection of PrP in a range of mammalian species and that the use of chickens as producers of mAbs may make it possible to produce anti-PrP antibodies of even greater specificity.

Acknowledgments

We thank Dr. D. Higgins of University of Hong Kong for critical review of the manuscript. This work was partly supported by a Grant-in-Aid for Exploratory Research from the Ministry of Education, Science and Culture, a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare and a Grant-in-Aid (Research Project for Studies on Pathogenesis of Prion Disease) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

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