Construction of recombinant monoclonal antibodies from a chicken hybridoma line secreting specific antibody

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Abstract

The chicken is a useful animal for the development of the specific antibodies against the mammalian conserved proteins. We generated two types of recombinant chicken monoclonal antibodies (mAbs), using a phage display technique from a chicken hybridoma HUC2-13 which secreted the mAb to the N-terminal of the mammalian prion protein (PrP). Although the mAb HUC2-13 is a useful antibody for the prion research, the hybridoma produces a low level of antibody production. In order to produce a large amount of the mAb, we have constructed a single chain fragment variable region (scFv) mAb by using the variable heavy (V_H) and light (V_L) genes which were amplified by using the two primer pairs and the flexible linker. The two phage display mAbs (HUC2p3 and HUC2p5) expressed on a M13 filamentous phage and their soluble type mAbs (HUC2s3 and HUC2s5) were reacted with the PrP peptide antigen in the ELISA. In the Western blot analysis, the mAbs HUC2p3 and HUC2s3 were as reactive to PrP^C from mouse brains as the mAb HUC2-13 was. The nucleotide sequences of V_H and V_L genes from HUC2-13 and the two clones were identical except for only one residue. These results indicate that the methods presented here provide an effective tool for the improvement of the low levels of antibody production in the chicken hybridoma system.

Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenyl; FBS, fetal bovine serum; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PBS, phosphate buffered saline; PBST, PBS containing 0.05% Tween 20; PrP^C, cellular prion protein; PrP^{Sc}, abnormal isoform of PrP^C; scFv, single chain fragment variable region.

Introduction

The chicken monoclonal antibody (mAb), HUC2-13, was generated from a fusion which used a chicken fusion partner cell line, MuH1 (Nishinaka et al., 1996), and the spleen cells from a chicken immunized with H25 peptide, 25th to 49th amino acid residues of the human prion protein (PrP) coupled with KLH

(Matsuda et al., 1999). The mAb reacted with the N-terminal (RPKPG-sequence, 25th to 29th amino acid residues) of the mammalian conserved region (RPKPG-sequence) of the H25 peptide and immunostained specifically the mammalian PrP in the Western blot and the immunohistochemical analyses. The reactivity of the mAb HUC2-13 to PrP materials was superior to the conventional PrP-specific mouse mAb

3F4 (Kascsak et al., 1987) which was widely used in prion research, in background staining (Matsuda et al., 1999). In these analyses the undiluted culture supernatant of the HUC2-13 hybridoma cells was used because the antibody concentration of the culture was low which was true for most of the chicken hybridomas established by us (Nishinaka et al., 1991, 1996; Asaoka et al., 1992; Matsushita et al., 1998). For further applications of the mAb HUC2-13, the preparation of a large amount of the mAb was required.

Recently, two different groups have reported the construction of the phage display chicken mAbs using the heavy and light variable regions (V_H and V_L) of the antibody genes from the bursa of Fabricius (Davies et al., 1995) and the spleen (Yamanaka et al., 1996) in the chicken.

In the construction of the phage display antibody, there are three different methods. The first method is the selection of the specific antibody by shuffling of the V_H and V_L genes using the lymphoid cells from a non-immunized animal (Gram et al., 1992; Davies et al., 1995). The second method is using the lymphoid cells from an immunized animal (Clackson et al., 1991; Williamson et al., 1996; Yamanaka et al., 1996) and the third method is using the specific hybridomas (Bell et al., 1995; Dziegiel et al., 1995; Yamanaka et al., 1995). The third method is more useful than the other methods when specific hybridomas are already established. In this study, therefore, we constructed phage display and soluble scFv mAbs were produced by using hybridoma cells which produce the mAb HUC2-13. In this article, we present the antigen-binding characteristic of the phage display and soluble scFv mAbs.

Materials and methods

Chicken hybridoma line

The chicken hybridoma cells, which produce the specific mAb, HUC2-13, to the N-terminal sequence (RPKPG) of PrP is conserved in many kinds of mammals (Matsuda et al., 1999). The cells were routinely maintained in an Iscove's modified Dulbecco's medium which contained 10% fetal bovine serum in a 5% CO₂ incubator at 38.5 °C.

RT-PCR

Total RNA was extracted from the HUC2-13 hybridoma cells by using ISOGEN-LS (Nippon Gene,

Japan). The 1st strand cDNA primed by Oligo dT primer (Boehringer Mannheim, Germany) was synthesized by using a Superscript II synthesis cDNA kit (GIBCO BRL, MD). The V-regions of the mAb were amplified by PCR with KOD DNA polymerase (TOYOBO, Japan) (Takagi et al., 1997) using the two primer pairs, CHB and CHSF for V_H and CLSB and CLF for V_L (Table 1) (Yamanaka et al., 1996). After one cycle of the PCR at 98 °C × 10 s, the V_H and V_L genes were amplified by 30 cycles of PCR at 98 °C × 15 s, 60 °C × 7 s, 74 °C × 20 s and 74 °C × 30 s with the Mg²⁺ at a concentration of 6 mM. The scFv linker was prepared by using the PCR of pLINK (Huston et al., 1988; Yamanaka et al., 1995), and by using the primers, 5'-TCATCGTGTGCGACAGGTGGAGG-3' and 5'-AGTCAGCGCGACGTCAGAACCT-3'. One hundred ng of purified V_H and V_L fragments was assembled with 20 ng of purified scFv linker by the PCR in order to get the scFv fragment (Yamanaka et al., 1995). The reamplification of the scFv was performed by using primers, CRB and CRF (Table 1).

Sequencing

The nucleotide sequencings of the cloned scFv were performed by the partial modification of a method described previously (Yamanaka et al., 1996). The cloned scFv genes were analyzed with the M13 reverse or the SLP-1 primer (Yamanaka et al., 1995) by using a dideoxy terminator method which included a Thermo Sequenase II terminator cycle sequencing kit (Amersham Pharmacia Biotech, Sweden) and an automatic DNA sequencer 373S (PE Applied Biosystems, CA).

Cloning vector pPDS

We used the pPDS (Yamanaka et al., 1995) as a cloning vector which has been designed to express the mouse scFv or the Fab of the C-terminal half of coat protein III (cp3) of the filamentous M13 phage under the control of the lactose promoter and operator of pBluescriptII. The ribosomal binding site and the leader sequence of cp3 are followed by the restriction sites of *EagI* and *BssHII* for cloning. In order to produce the soluble antibody fragment, the vector is inserted to the stop codon TAG between the mouse C_k and the cp3 (Figure 1).

Construction of scFv

Two hundred ng of the scFv fragments was ligated with 1 µg of pPDS at the *EagI* and *BssHII* sites. The

Table 1. Primers used for PCR amplification of chicken immunoglobulin variable regions¹

CHB 5'-CTGATGGCGGCCGTGACGTT-3'	CHSF 3'-TGGCTTCAGTAGCACAGCTGTCCACCT-5'
CLSB 5'-TCTGACGTCGCGCTGACTCAGCC-3'	CLF 3'-TTGGGACTGGCAGGAATTCGCGCGATTA-5'
CRB 5'-TATCTGATGGCGGCCGTGA-3'	CRF 3'-GGAATTCGCGCGATTATATCAG-5'

¹ CHB, reverse primer for heavy chain variable region. CHSF, forward primer for heavy chain. CLSB, reverse primer for λ chain. CLF, forward primer for λ chain. CRB, reverse primer for reamplification of scFv fragment. CRF, forward primer for reamplification of scFv fragment.

Figure 1. Structure of cloning vector pPDS (Yamanaka et al., 1995). RBS+g3L: ribosomal binding site and leader sequence of cp3; C κ : mouse C κ sequence.

resulting DNA was transformed into 100 μ l aliquots of *Escherichia coli* XL1-Blue competent cells, and then plated on SOB agar supplemented 100 mg ml⁻¹ of ampicillin and 0.2 M glucose. The culture was then infected with VCS-M13 (7.5×10^9 PFU) in a medium containing ampicillin, tetracycline and glucose. The cells were collected by centrifugation and resuspended in 5 ml of SuperBroth containing ampicillin, tetracycline and kanamycin, and then IPTG was added at a final concentration of 250 μ M. The culture was incubated overnight at 37 °C while shaking vigorously. The recombinant phage was collected by centrifugation. The soluble scFv antibody which was prepared by using non-amber suppressor cells, SOLR strain (Stratagene, CA, USA) was obtained as the bacterial culture supernatants and the supernatant from the bacteria sonicated with Ultrasonicator (Tomy, Japan).

Figure 2. Comparison of PCR profiles between HUC2-13 and 3C8 in their V_H, V _{λ} and scFv. Lane 1, pUC118 *Hinf*I digests; lane 2, V_H of HUC2-13; lane 3, V_H of 3C8; lane 4, V _{λ} of HUC2-13; lane 5, V _{λ} of 3C8; lane 6, scFv of HUC2-13; lane 7, scFv of 3C8; lane 8, ϕ x174 *Hae*III digests. The PCR products were separated on 1.5% agarose gel. The numbers at the both sides represent the apparent molecular masses in bp.

ELISA

Each wells of the ELISA plates was coated with 50 μ l of 10 μ g ml⁻¹ of H25 peptide (Human prion protein peptide, 25th to 49th amino acid residues). Then, the ELISA plate was blocked with 50% BlockAce (Yukijirushi, Japan) in PBS at 37 °C for 2 hr. The scFv mAb mixed with BlockAce, reacted with the solid-phase antigen for 2 hr at 37 °C. The bound mAb was detected by using peroxidase-labelled anti-M13 antibody (Amersham Pharmacia Biotech, Sweden) for the phage display mAb, alkaline phosphatase-labelled anti-mouse Ig antibody (Kirkegaard and Perry Laboratories, MD, USA) or alkaline phosphatase-labelled anti-mouse κ antibody (Southern Biotechnology, AL, USA) for the soluble mAb. The color development for the phage display antibody was done by using *o*-phenyldiamine to measure the absorbance at 492 nm. The reaction of the soluble antibody was

Figure 3. Binding specificity of two chicken phage display mAbs, HUC2p3 and HUC2p5. Solid bars represent H25 peptide. Open bars represent DNP₂₀-KLH antigen. Striped bars represent DNP₂₀-BSA antigen. The 3C8 mAb (Yamanaka et al., 1996) and the pPDS without insert were used as negative controls. In this experiment, the OD value (OD₄₀₅) for the supernatant from the HUC2-13 hybridoma culture as a positive control was 1.854 ± 0.064 .

visualized by using *p*-nitrophenyl phosphate to measure the absorbance at 405 nm. The optical density measurements were made using a Microplate Reader MPR A4 (TOSOH, Japan). The culture supernatant of the HUC2-13 hybridoma cells was used as a positive control antibody, whereas the phage display or soluble chicken scFv antibody 3C8, specific to murine serum albumin (MSA), (Yamanaka et al., 1996) and the pPDS vector without any inserts were also used as the negative control antibodies. The two kinds of antigens, DNP₂₀-KLH and DNP₂₀-BSA, were also used as the negative control antigens.

Western blotting

In order to obtain the PrP^c from the mouse, we prepared the membrane fraction of the brain of a normal BALB/c mouse. The brain tissue (200 mg) was homogenized in 2 ml of a 10 mM Tris-HCl/100 mM NaCl/proteinase inhibitor cocktail (Boehringer Mannheim, Germany). The homogenate was centrifuged at $800 \times g$ for 5 min. The supernatant was centrifuged again at $100\,000 \times g$ for 60 min. The resulting pellet was boiled in a 1% SDS solution, and the protein concentration was measured by a BCA kit (Pierce, IL, USA). For the Western blotting, we ran the PrP samples (50 μ g protein content per lane) in a 15% polyacrylamide gel. The procedures for the mem-

Figure 4. Binding specificity to H25 peptide of two chicken soluble mAbs, HUC2s3 and HUC2s5. The 3C8 mAb (Yamanaka et al., 1996) was used as a negative control.

brane transfer and the immunostaining were described elsewhere (Kitamoto et al., 1992).

In biochemical analysis of the soluble scFv antibody, the supernatants of the sonicated bacteria were respectively assessed by SDS-PAGE with coomassie brilliant blue (CBB) staining and immunoblot analysis using alkaline phosphatase-labelled anti-mouse κ antibody (Southern Biotechnology, AL, USA).

Results and discussion

Construction of HUC2-13-derived scFv

The mechanism of molecular diversification of Ig in the chicken is distinct from that in mammals. In mammals the preimmune repertoire is constructed by combinatorial and junctional diversity which is generated by the V-(D)-J recombination (Bernard et al., 1978; Sakano et al., 1980). In chicken λ chains, the contribution of the V-J recombination is not significant, since there is only one functional V (V λ 1) and J (J λ) gene for the entire light chain repertoire (Reynaud et al., 1985, 1987; Thompson and Neiman, 1987). The preimmune repertoire of the chicken λ chain is mainly constructed by the gene conversion of a re-

Figure 5. Western blotting profiles of recombinant chicken mAbs, HUC2p3 and HUC2s3, for PrP^{Sc} from normal mouse brain (A) and HUC2s3 (B). Purified mouse PrP^{Sc} fractions were separated on the 15% SDS-polyacrylamide gel (A). Lane 1 was stained with supernatant from HUC2-13 hybridoma culture. Lane 2 was stained with phage display mAb HUC2p3. Lane 3 was stained with soluble mAb HUC2s3 (supernatant from the sonicated bacteria). Lane 4 was stained with recombinant mouse κ from pPDS as a negative control antibody. In the lanes 2 and 3, the positive stainings were obtained as well as the hybridoma culture supernatant (Lane 1). The soluble mAb HUC2s3 (supernatant from the sonicated bacteria) was separated on the 12.5% SDS-polyacrylamide gel (B). Lane 1 was stained with CBB and lane 2 was stained with alkaline phosphatase-labelled anti-mouse Ig. Arrows indicate three types of PrP molecules. The numbers at the left (A) and the right (B) represent the apparent molecular masses in kDa.

arranged functional V λ 1 gene with pseudo J λ genes clustering upstream of the functional gene as sequence donors (Reynaud et al., 1987; Thompson and Neiman, 1987). A similar mechanism operates in the H chain preimmune repertoire in which the genomic DNA composed of the unique functional V (V_H1) and J (J_H) genes, about 15 D genes, and about 90 pseudo V_H genes clustering upstream of V_H1 (Reynaud et al., 1989). Therefore, the 3' region of framework 4, which is derived from J λ or J_H, is highly conserved in its nucleotide sequences in the mature Ig mRNA. The 5' region of framework 1 is also conserved, since the gene conversion is not frequent there (McConnack and Thompson, 1990), and the sequences of the pseudo-genes corresponding to this region do not show much divergence (Reynaud et al., 1987). This makes it possible to perform a reverse transcription-PCR of the V region repertoire with a single pair of primers.

In this study, the cDNA generated from the V region mRNA, which were approximately 380 bp for the H chain and 330 bp for the λ chain, were amplified,

purified and assembled into the scFv fragments. The sizes of the H and λ chains and the scFv from the HUC2-13 were identical to those of a negative control 3C8 (Figure 2). As shown in the Materials and methods section, the KOD DNA polymerase which had a similar mutation frequency to the *pfu* DNA polymerase and a higher extension rate and processivity than the *pfu* DNA polymerase (Takagi et al., 1997), was used for the amplification of H and λ chains and obtained the satisfactory results. The amplification time by PCR of Ig chains could be reduced by about one-tenth by using the polymerase.

After ligation into the pPDS and the transformation into bacteria, a phage displaying HUC2-13 scFv was constructed by the infection with a helper phage, and the resulting two positive colonies, No. 3 and No. 5, were selected. These two scFv mAbs were provisionally named as HUC2p3 and HUC2p5, respectively. The phage titers of these positive clones were 5×10^{10} to 5×10^{11} CFU ml⁻¹. The soluble form of the scFv antibody was also prepared by using the non-amber suppressor cells. In this study, the bacterial culture supernatants were used as soluble scFv mAb, and referred as HUC2s3 and HUC2s5, respectively.

Specificity of HUC2-13-derived scFv

The specificity of the two phage display mAbs was determined by ELISA using several antigens, H25 peptide, DNP₂₀-KLH or DNP₂₀-BSA. About 0.7 to 3.0×10^{11} recombinant phage were applied to the antigen-coated plates. The OD₄₉₂ values of the two positive clones by the ELISA test were more than 1.4 to H25 peptide, whereas those of the pPDS without insert and control phage display mAb 3C8 to MSA were less than 0.3 (Figure 3). Further, the OD₄₉₂ values of all tested samples against the DNP antigens were less than 0.1.

The specificity of the soluble mAbs, HUC2s3 and HUC2s5, was also determined by ELISA using the H25-peptide (Figure 4). These two soluble mAbs specifically reacted with the antigen in spite of the use of bacterial supernatants, whereas the soluble mAb 3C8 negative control did not react with the antigen. When the soluble mAb from sonicated bacteria was used, the higher reactivity to the peptide was demonstrated (Figure 4).

The 3C8 mAb to MSA was made by using the pPDS vector and the immune chicken spleen cells with MSA (Yamanaka et al., 1996). The anti-MSA phage display mAb was finally selected by a three round pan-

A Heavy chains

Germline	<u>GCCGTGACGTTGGACGAGTC</u> CGGGGCGCGCTCCAGACGC CCGGAAGAGCGCTCAGCCTC GTCTGCAAGGCCTCCGGGTT 80
	Reverse primer
HUC2-13	-----G--G-----T-----
NO.3	-----A-----G--G-----T-----
NO.5	-----G--G-----T-----
Germline	CACCTTCAGCAGTTACAAACA <u>TGGGTTGGGTGCGACAGGCG</u> CCGGCAAGGGGCTGGAGTT CGTCGCTGGTATGACAACA 160
	CDR1
HUC2-13	-T-----CC-TGG-- --C-----C----- --T---GA-----G G---G-CT---AG-GGTG
NO.3	-T-----CC-TGG-- --C-----C----- --T---GA-----G G---G-CT---AG-GGTG
NO.5	-T-----CC-TGG-- --C-----C----- --T---GA-----G G---G-CT---AG-GGTG
Germline	<u>CTGGTAGATACACAGGCTAC</u> GGGTCGGCGGTGAAGGGCGG TGCCACCATCTCGAGGGACA ACGGGCAGAGCACAGTGAGG 240
	CDR2
HUC2-13	-----TAG--A-A-----C-----
NO.3	-----TAG--A-A-----C-----
NO.5	-----TAG--A-A-----C-----
Germline	-----VH1 gene-----/-----D segment-----
	CTGCAGCTGAACAACCTCAG GGCTGAGGACACCGGCACCT ACTACTGCGCCAAAGCTGCT GGTNNNNNNNNNNNNNNNN 320
	CDR3
HUC2-13	-----A-ACTGTTTACGATGATGA
NO.3	-----A-ACTGTTTACGATGATGA
NO.5	-----A-ACTGTTTACGATGATGA
Germline	-----/-----JH gene-----
	NNNNNNNNNNNNNACTGCTG <u>GTAGCATCGACGCATGGGGC</u> CACGGGACCGAAGTCATCGT CTCCTCC
	Forward primer
HUC2-13	TGATACTTATATA-G---- A-AT-----A-G----- G--GA-A
NO.3	TGATACTTATATA-G---- A-AT-----A-G----- G--GA-A
NO.5	TGATACTTATATA-G---- A-AT-----A-G----- G--GA-A

B Light chains

Germline	<u>GCGCTGACTCAGCCGTCCTC</u> GGTGTCAGCGAACCCGGGAG GAACCGTCAAGATCACCTGC <u>TCCGGGGATAGCAGTACTA</u> 80
	Reverse primer
HUC2-13	-----G-----A--T-----G-----:::-----
NO.3	-----G-----A--T-----G-----:::-----
NO.5	-----G-----A--T-----G-----:::-----
Germline	<u>TGGCTGGTACCAGCAGAAGG</u> CACCTGGCAGTGCCCTGTC ACTGTGATCTATGACAACAC CAACAGACCCCTCGAACATCC 160
	CDR2
HUC2-13	-----T -T-----T-----AG-----G-----
NO.3	-----T -T-----T-----AG-----G-----
NO.5	-----T -T-----T-----AG-----G-----
Germline	CTTCACGATTCTCCGGTTCC AAATCCGGCTCCACAGCCAC ATTAACCATCACTGGGGTCC GAGCCGACGACAATGCTGTC 240
HUC2-13	-----C-----AG-----A-----G--G-G-----
NO.3	-----C-----AG-----A-----G--G-G-----
NO.5	-----C-----AG-----A-----G--G-G-----
Germline	-----VL1 gene-----/-----JL gene-----
	TATTACTGTGCGAG:TACAG <u>ACAGCAGCAGTACTGCAGGT</u> ATATTGGGGCCGGGACAAC <u>CCTGACCGTCCTAGGT</u>
	CDR3 Forward primer
HUC2-13	---T---G---C---:---T---T---TA---T-A- C-C-----TAAG
NO.3	---T---G---C---:---T---T---TA---T-A- C-C-----TAAG
NO.5	---T---G---C---:---T---T---TA---T-A- C-C-----TAAG

Figure 6. Nucleotide sequences of V_H (A) and V_L (B) chains of HUC2-13-derived mAbs. Germline sequences of CB inbred strain (Reynaud et al., 1987; 1989) are shown at the top. Nucleotide identity to the germline sequences is shown by dash. The absence of corresponding residues is shown by colon. CDR and sequences to which PCR primers anneal are underlined. Sequences to be filled with D segment in the germline heavy chain are shown by Ns.

ning to a specific antigen. To the contrary, in this study, we obtained significant specific binding to the H25-peptide antigen without repeated panning. The result indicates that if the specific hybridomas are available, the phage display mAbs can be selected easier than by using the immune spleen cells. Further, the development of the phage display chicken mAbs using the non-immunized chickens (Davies et al., 1995) requires several panning steps.

The chicken mAb system using cell fusion has already been established by us (Nishinaka et al., 1991, 1996), but the antibody concentration of the supernatant in the chicken hybridoma culture is usually less than $5 \mu\text{g ml}^{-1}$ (Nishinaka et al., 1996; Matsuda et al., 1999). Therefore, for the further application of the chicken mAb, a higher concentration of the mAb is required. In this study, we constructed successfully the chicken phage display and the soluble type scFv mAbs from the specific chicken hybridoma. It is the first time that the recombinant chicken mAb could be constructed from the specific chicken hybridoma cells. Therefore, the results presented here indicate that recombinant antibody can be easily established if the specific hybridomas are available.

Western blotting

The specificity of the two mAbs, the phage display mAb HUC2p3 and the soluble mAb HUC2s3 from the sonicated bacteria, derived from the positive clone No. 3, was determined by Western blotting using the PrP^c from normal mouse brains (Figure 5A). These two recombinant mAbs as well as the culture supernatant from the HUC2-13 hybridoma reacted specifically with the PrP^c molecules. However, no the soluble material (mouse C κ) from the pPDS vector without any inserts as the negative control antibody was reacted with the molecules.

For the biochemical analysis of the HUC2s3, SDS-PAGE and Western blotting using the supernatant of the sonicated bacteria were done. Many bands were visible in CBB-staining of the material, whereas the recombinant antibody HUC2s3 was immunostained as a single band of apparent molecular weight of 45 kDa in agreement with the predicted size of the HUC2s3 including mouse C κ ,

Nucleotide sequences of scFv mAbs

Two positive scFv clones, No. 3 and No. 5, were sequenced. The nucleotide sequences of these two clones were identical with each other except for only

one residue in the V_H sequence of No. 3. Furthermore, the V_H and V λ sequences of No. 5 were identical to the sequence from the HUC2-13 hybridoma cells (Figure 5). The sequence differences between the germline and the HUC2-13 and the two clones were predominantly in the CDR regions, as expected. However, the change in the V λ -CDR1 region of the HUC2-13 and the two clones was only the absence of tri-nucleotide residues. By comparing λ -chain nucleotide sequences with 25 pseudo V λ genes of the CB chicken strain (Reynaud et al., 1987) and other known pseudo V λ genes in different chicken strains (Kondo et al., 1993; Benatar et al., 1993), we concluded that the absence of the tri-nucleotide residues in the V λ -CDR1 region was derived from the pseudo genes, $\psi 2$ or $\psi 18$.

Conclusions

The results presented here demonstrate that the chicken antibodies can be effectively constructed as the phage display and soluble mAbs from the specific hybridoma HUC2-13. Although many kinds of chicken hybridomas which secrete the specific antibodies have been established by using cell fusion with the immune spleen cells from chickens and fusion partner cell lines, the antibody production by these hybridomas was less than about one-tenth of the mouse system in many cases (Nishinaka et al., 1991, 1996; Asaoka et al., 1992; Matsushita et al., 1998; Matsuda et al., 1999). Further, since these fusion partner lines were derived from the outbred chicken line, the chicken hybridomas could not make ascites (data not shown). However, these problems will be improved by the technique for making the recombinant chicken scFv mAbs reported here.

Because the cloning vector pPDS was first constructed for making the mouse Fab mAb, the vector contains the mouse C κ sequence (Yamanaka et al., 1995). Therefore, the sequence of the chicken scFv is constructed as the chicken V_H-linker-chicken V λ -mouse C κ . Although it is possible to use the chicken scFv mAb for antigen detection, if the mAb use in the two antibody assay system together with the mouse mAb, the use may be restricted. For the further application of the chicken scFv, the construction of a new cloning vector containing the chicken C λ for making the chicken scFv mAb is now in progress.

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References

- Asaoka H, Nishinaka S, Wakamiya N, Matsuda H & Murata M (1992) Two chicken monoclonal antibodies specific for heterophil Hanganutziu-Deicher antigens. *Immunol Lett* 32: 91–96.
- Bell CW, Scholthof KB, Zhang G & Karu AE (1995) Sequences of cDNAs encoding the heavy- and light-chain Fab region of an antibody to the phenylurea herbicide diuron. *Gene* 165: 323–324.
- Benatar T & Ratcliffe MJH (1993) Polymorphism of the functional immunoglobulin variable region genes in the chicken by exchange of sequence with donor pseudogenes. *Eur J Immunol* 23: 2448–2453.
- Bernard O, Ozumi N & Tonegawa S (1978) Sequences of murine immunoglobulin light chain genes before and after somatic changes. *Cell* 15: 1133–1144.
- Clackson T, Hoogenboom HR, Griffiths AD & Winter G (1991) Making antibody fragments using phage display libraries. *Nature (Lond.)* 352: 624–628.
- Davies EL, Smith JS, Birkett CR, Manser JM, Anderson-Dear DV & Young JR (1995) Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *J Immunol Methods* 186: 125–135.
- Dziegiel M, Nielsen LK, Andersen PS, Blancher A, Dickmeiss E & Engberg J (1995) Phage display used for gene cloning of human recombinant antibody recombinant antibody against the erythrocyte surface antigen, rhesus D. *J Immunol Methods* 182: 7–19.
- Gram H, Marconi LA, Barbas CF, Collet TA, Lerner RA & Kang AS (1992) In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc Natl Acad Sci USA* 89: 3576–3580.
- Huston JS, Levinson D, Mudgett-Hunter M, Tai M-S, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R & Oppermann H (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 85: 5879–5883.
- Kascak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM & Diringier H (1987) Mouse polyclonal and monoclonal antibodies to scrapie-associated fibril proteins. *J Virol* 61: 3688–3693.
- Kitamoto T, Shin R-W, Doh-ura K, Tomokane T & Tateishi J (1992) Abnormal isoform of prion proteins accumulates in the synaptic structures of the central nervous system in patient with Creutzfeldt-Jakob disease. *Am J Pathol* 140: 1285–1294.
- Kondo T, Arakawa H, Kitao H, Hirota Y & Yamagishi H (1993) Signal joint of immunoglobulin V λ 1-J λ and novel joints of chimeric V pseudogenes on extrachromosomal circular DNA from chicken bursa. *Eur J Immunol* 23: 245–249.
- Matsuda H, Mitsuda H, Nakamura N, Furusawa S, Mohri S & Kitamoto T (1999) A chicken monoclonal antibody with specificity for the N-terminal of human prion protein, FEMS Immunol and Med Microbiol 23: 189–194.
- Matsushita K, Horiuchi H, Furusawa S, Horiuchi M, Shinagawa M & Matsuda H (1998) Chicken monoclonal antibodies against synthetic bovine prion protein peptide. *J Vet Med Sci* 60: 777–779.
- McCormack WT & Thompson CB (1990) Chicken IgL variable region gene conversions display pseudogene donor preference and 5' to 3' polarity. *Genes Dev* 4: 548–558.
- Nishinaka S, Akiba H, Nakamura M, Suzuki T, Tsubokura K, Horiuchi H, Furusawa S & Matsuda H (1996) Two chicken B cell lines resistant to ouabain for the production of chicken monoclonal antibodies. *J Vet Med Sci* 58: 1053–1056.
- Nishinaka S, Suzuki T, Matsuda H & Murata M (1991) A new cell line for production of chicken monoclonal antibody by hybridoma technology. *J Immunol Methods* 139: 217–222.
- Reynaud C-A, Anquez V, Dahan A & Weill J-C (1985) A single rearrangement event generates most of the chicken immunoglobulin light chain diversity. *Cell* 40: 283–291.
- Reynaud C-A, Anquez V, Grimal H & Weill J-C (1987) A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* 48: 379–388.
- Reynaud C-A, Dahan A, Anquez V & Weill J-C (1989) Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell* 59: 171–183.
- Sakano H, Maki R, Kurosawa Y, Roeder W & Tonegawa S (1980) Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature* 286: 676–683.
- Takagi M, Nishioka M, Kakiyama H, Kitabayashi M, Inoue H, Kawakami B, Oka M & Imanaka T (1997) Characterization of DVA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. *Appl Environ Microbiol* 63: 4504–4510.
- Thompson CB & Neiman PE (1987) Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell* 48: 369–378.
- Williamson RA, Peretz D, Smorodinsky N, Bastidas R, Serban H, Mehlhorn I, DeArmond SJ, Prusiner SB & Butron DR (1996) Circumventing to tolerance to generate autologous monoclonal antibodies to the prion protein. *Proc Natl Acad Sci USA* 93: 7279–7282.
- Yamanaka HI, Kirii Y & Ohmoto H (1995) An improved phage display antibody cloning system using newly designed PCR primers optimized for *pfi* DNA polymerase. *J Biochem* 117: 1218–1227.
- Yamanaka HI, Inoue T & Ikeda-Tanaka O (1996) Chicken monoclonal antibody isolated by a phage display system. *J Immunol* 157: 1156–1162.