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Selection and affinity maturation of human antibodies against rabies virus from a scFv gene library using ribosome display

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ABSTRACT

We selected useful antibody fragments against rabies virus from a human single chain variable fragment (scFv) gene library using ribosome display technique. The recombinant rabies virus glycoprotein (RVGp) was used as an antigen to isolate specific scFvs. After five rounds of selection, the analysis demonstrated that scFv–ribosome–mRNA complexes were specifically selected against RVGp. Sequence analysis showed that mutations were introduced at random by PCR among the rounds of selection and variants with high affinity were isolated. The obtained scFvs with high affinity could recognize RVGp specifically and showed binding activity to rabies virus. These scFvs were potential for inclusion in a combination of several human monoclonal antibodies (MAbs) aimed for application in rabies post-exposure prophylaxis. Ribosome display technology is a robust tool for rapid isolation of human antibody fragments, and has exceptional strength in affinity maturation and molecular evolution in vitro.

1. Introduction

Rabies is a worldwide zoonosis caused by rabies virus. Effective post-exposure treatment of rabies, as recommended by the World Health Organization, includes the prompt usage of human or equine anti-rabies immunoglobulin (RIG), and the administration of rabies vaccine (World Health Organization, 2002). The urge to replace these hyper-immune serum preparations is widely recognized. A combination of several human monoclonal antibodies (MAbs) that can neutralize rabies virus offers the opportunity to replace RIG (Hanlon et al., 2001; Sun et al., 2007). Therefore, we initiated a study to identify and characterize human MAbs that are compatible with each other for potential application in rabies post-exposure prophylaxis.

Display technology provides powerful and effective approaches to discovery and selection of proteins in vitro, especially antibodies. The key feature of all display technologies is the physical linkage between polypeptide and its corresponding nucleic acid sequence. Display technologies allow one to bypass in vivo immunizations of animals or human beings to produce antibodies which could be used for inclusion in antibodies combination. Phage display is the most widely used display technique to identify human antibody fragments with specific binding activities. To prepare antibodies against rabies virus, we previously selected specific single chain variable fragments (scFvs) to G5-peptide by phage display. G5 is a linear epitope on rabies virus glycoprotein (Gp), which is responsible for inducing and binding of neutralizing antibodies. However, the selected scFvs can only interact with G5-peptide in vitro (Zhao et al., 2008). This maybe result from the following two reasons, firstly, G5-peptide was poorly immunogenic; secondly, the library size used in our study was insufficiently large for efficient display and selection of antibodies. Like other cell-dependent methods, phage display suffers a potential limitation in library size, diversity, protein expression and secretion in *Escherichia coli* (Groves et al., 2006; Matsuura and Plückthun, 2003).

Newly developed ribosome display technology has some outstanding advantages and is more effective than phage display. Ribosome display is performed completely in vitro (in a cell-free system); it circumvents transformation and the associated inefficiencies (Schaffitzel et al., 1999; Jermutus et al., 2001). Moreover, as a PCR based technique, ribosome display provides a faster and more efficient selection method than phage display screening (Hanes and Pluckthun, 1997; Hanes et al., 2000; He and Taussig, 1997). By ribosome display technology, this study was conducted to select useful antibody fragments against rabies virus from a phage display scFv gene library.

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2. Materials and methods

2.1. Construction of ribosome display repertoires

A scFv gene library for phage display (constructed from the peripheral blood lymphocytes of three donors immunized with rabies virus vaccine by Zhao et al., 2004) was converted to ribosome display format by PCR as follows. The scFv genes were amplified using Taq polymerase (TakaRa) with 25 cycles of PCR (one cycle consists of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). In the procedure, two primers were used, namely, RB/back that contained a T7 promoter region: 5′-ata cga aat taa tac gac tca cta tag gga tg(c/g) agg t(g/c)c a(g/c)c tcg ag(c/g) agt ctg g-3′, and RB/for which had no stop codon: 5′-tgc agc cac agt acg ttt gat ctc ca-3′. A spacer, derived from amino acids 211–299 of gene III of filamentous phage M13 mp19 (Hanes and Pluckthun, 1997), was amplified using gene III specific primers, S/back: 5′-gct gat gct gca ggc ggt t-3′, S/for: 5′-ccg cac acc agt aag gtg tgc ggt atc acc agt agc acc-3′. The primary PCR products were purified with gel extraction kit (QIAGEN). After gel purification, 15 ng scFv DNA and 25 ng spacer DNA were mixed with 25 μl PCR mixtures and amplified with 7 cycles (one cycle consists of 1 min at 94 °C, 2 min at 60 °C, and 2 min at 72 °C). The assembled DNA was amplified in 50 μl PCR mixture for 25 cycles (one cycle consists of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C) with RB/back and S/for primers. PCR products were used for transcription in vitro.

2.2. In vitro transcription and translation

The mRNA transcripts were obtained by in vitro transcription using the purified PCR products with T7 RNA polymerase (Promega, USA). Briefly, about 1 μg library DNA was added to 50 μl transcription mixture containing 40 mmol/L Tris–HCl (pH 7.9), 6 mmol/L $MgCl_2$, 2 mmol/L spermidine, 10 mmol/L NaCl, 10 mmol/L DTT, 50 U RNase inhibitor, 0.5 mmol/L rNTP and 20 U T7 RNA polymerase. The mixture was incubated at 37 °C for 2 h and the reaction was stopped by phenol/chloroform extraction. The transcripts were purified by 3 mol/L LiCl, and then dissolved in DEPC (diethylpyrocarbonate) treated water.

In vitro translation was performed in 70% nuclease-treated reticulocyte lysate (Promega, UAS). The reaction volume was 50 μl and the reaction mixture contained 2 μg purified mRNA transcripts, 35 μl rabbit reticulocyte lysate (nuclease treated), 1 μl amino acid mixture (complete, 1 mmol/L), 20 U RNase inhibitor. The translation was carried out at 30 °C for 30 min, then stopped by cooling on ice and adding 150 μl ice-cold PBSMB buffer (PBS with 5 mmol/L $MgCl_2$ and 5% BSA) immediately (Douthwaite et al., 2006).

2.3. Affinity selection

Micro-titer plates were coated at 4 °C overnight with 50 μl purified recombinant RVGp (provided by the lab of stress medicine, Institute of Health and Environmental Medicine, Tianjin, China) solution (1 μmol/Lin PBS). The coated plates were washed with PBS and blocked with sterilized 10% (w/v) skim milk in PBSM buffer (PBS with 5 mmol/L $MgCl_2$) for 30 min at room temperature. After being washed with PBS, the plates were blocked again with blocking buffer (5%BSA in PBSM) for 2 h. Then the plates were washed with PBSM three times and incubated on ice for at least 10 min.

The translation mixture was added to the prepared PBS-coated micro-titer well and incubated for pre-binding. After pre-binding, the supernatant was transferred to RVGp-coated well. The plate was incubated on ice for 1 h. After three washes with ice-cold PBSTM (PBS containing 5 mmol/L $MgCl_2$ and 0.05% Tween 20R) and two washes with ice-cold PBSM, the retained scFv–ribosome–mRNA complexes were dissociated with 200 μl EB20 buffer (PBS containing 20 mmol/L EDTA) for 10 min on ice. The mRNA was isolated from the eluted solution using an RNA isolation kit (QIAGEN).

2.4. RT-PCR

Selected mRNA was reverse transcribed to cDNA using transcriptase (Invitrogen). Using Taq DNA polymerase (TaKaRa), the obtained cDNA was amplified in 50 μl PCR mixture for 30 cycles (one cycle consists of 20 s at 95 °C, 40 s at 55 °C, 1 min at 68 °C) with RB/back and S/for primers. A Taq DNA polymerase lacking 3′ to 5′ exonuclease proofreading activity was used in the PCR procedure.

2.5. Cloning and expression

On behalf of scFv's expression and its analysis by ELISA, outputs from ribosome display were amplified by PCR using the primers RB/back NC 5′ gcc atg gcc atg (c/g)ag gt(g/c) ca(g/c) c 3′, RB/for NO 5′ gag tca ttc tgc ggc cgc tgc agc atc agc ccg ttt 3′ (the restriction sites NocI and NotI are underlined, respectively). The PCR was performed with pfu DNA polymerase (TaKaRa) for 25 cycles (one cycle consists of 30 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C). The amplified scFv DNA and pET22b(+) vector were digested with NocI and NotI, and then were purified by gel extraction kit (QIAGEN). Ligations of prepared insert DNA and pET22b(+) vector were carried out using T4DNA ligase (TaKaRa). The ligations were transformed into *E. coli* BL21 (DE3) and soluble proteins were expressed from each clone. Briefly, each single clone was cultured in 5 ml LB medium with 100 mg/ml ampicillin and 0.1% (w/v) glucose at 30 °C with 250 rpm shaking until they reached an absorbance of 0.7 at 600 nm. IPTG (isopropyl-b-D-thiogalactopyranoside) was added to a final concentration of 1 mmol/L, and the bacteria were incubated at 30 °C overnight with shaking at 150 rpm. The bacteria were pelleted and resuspended in 0.5 ml ice-cold 1× TES buffer (0.2 mol/L Tris–HCl [pH 8.0], 0.5 mmol/L EDTA, 0.5 mol/L sucrose) and 0.75 ml ice-cold 1/4× TES buffer. After incubation on ice for 30 min, the cells were pelleted by centrifugation at 10,000 rpm for 10 min. The scFv fragments was purified from the supernatant by affinity chromatography using a His Trap HP column (GE, CA, USA). The binding buffer was 20 mM phosphate buffer (pH 7.4) with 20 mM imidazole and 500 mM NaCl. ScFv fragments were eluted using phosphate buffer with imidazole at different concentrations (50, 100, 200, 300, 400 and 500 mM). The eluted fractions were concentrated by centrifugal filters (Millipore, Bedford, MA, USA).

2.6. ELISA

ELISA was performed routinely to identify the binding activity of the isolated clones. The plates were coated with 100 μl RVGp (40 μg/ml in PBS) overnight at 4 °C. After blocking with PBSB (PBS containing 5% BSA), 100 μl periplasmic extracts diluted 1:1 with PBSB were added to antigen-coated well and then incubated for 1 h at RT. Anti-His tag antibody (1:1000, Amersham) and horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG (1:5000, Jackson) were used as primary and secondary antibody in tandem. The results of absorbance were detected by spectrophotometer at wavelength of 405 nm. The positive clones were sequenced. The sequence was analyzed by DNA plot software and the Vector NTI software (Retter et al., 2005).

2.7. Immunoblot analysis of scFvs expression

The selected anti-RVGp scFv fragments were subjected to SDS-PAGE with 12% polyacrylamide gel. After SDS-PAGE, the gel was transferred to nitrocellulose membrane (Sigma). The transblotted membrane was blocked with blocking solution (2% [w/v] skim