**Original Article**

# Preparation and Initial Application of a Monoclonal Antibody Specific for a Newly Discovered Conserved Linear Epitope of Rabies Virus Nucleoprotein*

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**Abstract**

**Objective** To prepare monoclonal antibodies against a newly discovered and conserved linear epitope of *Rabies virus* nucleoprotein and to use them in a rabies diagnostic test.

**Methods** Synthetic peptide containing the epitope was used as immunogen to prepare hybridoma cell lines by classical hybridoma technology. Anti-peptide monoclonal antibodies produced in ascites of inoculated Balb/c mice were labeled with fluorescein isothiocyanate (FITC) after purification and used in fluorescent antibody test (FAT).

**Results** Two positive hybridoma cell lines, RVNP-mAb1-CL and RVNP-mAb2-CL, were obtained. RVNP-mAb1-CL produced a higher concentration of monoclonal antibody RVNP-mAb1 in Balb/c ascites. FITC-labeled RVNP-mAb1 showed correct results on certain *Rabies virus*-positive canine brain tissue samples and cells of a small subclone of baby hamster kidney 21 cell line (BSR).

**Conclusion** FITC-labeled RVNP-mAb1 has potential application for laboratory diagnosis of rabies.

**Key words:** *Rabies virus*; Nucleoprotein; Epitope; Peptide; Monoclonal antibody

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## INTRODUCTION

Sensitive and efficient fluorescein isothiocyanate (FITC) labeled monoclonal antibodies against *Rabies virus* nucleoprotein are critical factors for successful detection of *Rabies virus*-positive samples by fluorescent antibody test (FAT)[1]. Generally, an integrated *Rabies virus* or recombinant *Rabies virus* nucleoprotein expressed in prokaryotic[2-3] or eukaryotic[4-5] cells is used as an immunogen to prepare monoclonal antibodies against *Rabies virus*. With this approach, identifying epitopes of monoclonal antibodies requires a large amount of work[2]. World Health Organization (WHO) recommends anti-*Rabies virus* nucleoprotein monoclonal antibodies from Fujirebio Diagnostics Inc. and Millipore as rabies diagnostic reagents. However, these reagents are expensive and scarce. In countries

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with a high prevalence of rabies, homemade anti-*Rabies virus* nucleoprotein monoclonal antibodies should be accessible to insure availability for rabies laboratory tests[6-8].

The *Rabies virus* nucleoprotein domain containing residues 152-164 was identified as a conserved linear epitope[9]. Monoclonal antibodies against this epitope may therefore have the potential to recognize native *Rabies virus* nucleoprotein and find use as a rabies diagnostic reagent[10-11]. Synthetic peptide corresponding to residues 152-164 could be used as an immunogen to prepare monoclonal antibodies by classical hybridoma technology[10-11]. Identifying epitopes was simplified since the targeted epitope was known in advance[11]. We describe here a monoclonal antibody against *Rabies virus* nucleoprotein prepared using conjugated peptide as an immunogen and applied in laboratory diagnostic tests.

## MATERIALS AND METHODS

### *Peptide Synthesis and Keyhole Limpet Hemocyanin (KLH) Conjugation*

Peptide synthesis was conducted by $A^+$ Peptide Ltd. (Shanghai, China) using an ABI 433 peptide synthesizer. A cysteine residue was added to the N-terminus, resulting in the following sequence: CKISGQNTGNYKTN. In total, 10 mg of peptide was synthesized and aliquoted at 5 mg/tube. Peptide conjugation was conducted using an Imject® Maleimide Activated Immunogen Conjugation with mcKLH and the BSA Kit from Pierce (Rockford, Illinois, USA) according the manufacturer's instruction. KLH was mixed with succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and rotated at room temperature for 30 min to fully activate KLH. The mixture was purified using Sephadex G-25 column from Pharmacia (Peapack, New Jersey, USA) equilibrated with peptide conjugating buffer. Two elution peaks were detected by optical density (OD) and collected. The second elution peak containing excessive SMCC was discarded. The peptide was added to activated KLH and rotated at room temperature for 2 h. Over ninety-five percent of the peptide was conjugated. The conjugated peptide was purified after desalination and freeze-dried. The product was stored at -80 °C. The mass of freeze-dried powder used was equivalent to that of the peptide.

### *Animal Immunization and Anti-peptide Antibody Detection by Indirect ELISA*

Two 6-week-old adult BALB/c mice with body weights of approximately 18 g were provided by the Experimental Animal Center, Chinese Academy of Medical Sciences. A simplified immunization procedure was adopted[12]. For the initial immunization and first and second booster, 50 μg peptide was mixed with 10 μg complete Freund's adjuvant. For the last booster, 100 μg peptide was used. The mixture or peptide was dissolved in 200 μL 0.01 mol/L phosphate-buffered saline (PBS, pH 7.2), and the solution injected into the celiac of each mouse. The interval between two ordinal immunizations was three weeks.

One week after the first and second boosters, and 3 days after the last booster, 0.02 mL of blood was collected via the tail vein. The serum was separated, and anti-peptide antibody titer in each sample was measured by indirect ELISA[12]. Ninety-six-well plates were coated with 5 μg/well of synthetic peptide. Each serum sample was diluted from 1:1000 to 1:729000 by 3-fold serial dilutions. HRP-labeled goat anti-mouse IgG was used as a secondary antibody. The criteria for positive test results were ODpositive (OD for positive well) >0.1 and ODpositive/ODnegative (OD for negative well) $\geqslant$2.1.

### *Preparation of Hybridoma Cell Lines*

A mouse with an anti-peptide antibody titer greater than 1:10000 was selected for preparation of hybridoma cell lines according using standard hybridoma technology[12-13]. Indirect ELISA and indirect fluorescent assays (IFA) were conducted to detect anti-peptide antibodies in hybridoma supernatants[12]. For IFA, BSR cells infected with *Rabies virus* CVS-11 in 96-well plates were used to capture anti-peptide antibodies, FITC-labeled goat anti-mouse IgG was used as a secondary antibody. Positive clones in both tests were subcloned twice to ensure the stability of antibody secretion. Two BALB/c mice were intraperitoneally inoculated for every positive clone. The ascites was harvested and pooled. The monoclonal antibodies in the ascites were detected by IFA[9,12]. Two hundred microliters of ascites for every positive clone was diluted from 1:50 to 1:500 by increments of 50 using 0.01 mol/L PBS (pH 7.2). Hybridoma cell lines with high levels antibody secretion were selected for monoclonal antibody production.

### *Purification and Labeling of Monoclonal Antibodies*

Monoclonal antibodies were purified according to standard protocol[12] using DEAE-Sephadex A-50

filler from GE Healthcare (Fairfield, Connecticut, USA), and chromatography columns from Pharmacia. Fifty milliliters of ascites for every positive clone was loaded onto the column, and the flow-through collected. The flow-through was reloaded onto the column and equilibrated. The monoclonal antibody was eluted and dialyzed with 0.01 mol/L PBS (pH 7.2). Five micrograms of purified monoclonal antibody was used to assess the protein profile by 10% sodium dodecylsulfonate-polyacrylate gel electrophoresis (SDS-PAGE).

Purified monoclonal antibodies labeled with FITC (Sigma-Aldrich, St. Louis, Missouri, USA), and purified using Sephadex G-25 filler from GE, and chromatography columns from Pharmacia, according to the manufacturer's instructions. FITC and purified monoclonal antibodies were mixed for 2 h. The mixture was passed through Sephadex G-25 columns to remove unbound components. Monoclonal antibodies were quantified using a Beckman UC800 spectrophotometer (Beckman Coulter, Brea, California, USA) .

### *Initial Application of FITC-labeled Monoclonal Antibodies*

Four canine brain tissue samples, three *Rabies virus*-positive and one *Rabies virus*-negative, as previously diagnosed by LIGHT DIAGNOSTICS™ Rabies DFA 5100 Reagent at a 1:60 working dilution (Millipore, Billerica, Massachusetts, USA)[1], were used to validate the FITC-labeled monoclonal antibodies. The FITC-labeled monoclonal antibodies were diluted from 1:50 to 1:500 by increments of 50 using 0.01 mol/L PBS (pH 7.2) and used for FAT. In addition, the FITC-labeled monoclonal antibodies were used in rapid fluorescent foci inhibition tests (RFFIT) for 200 human serum samples, and compared with LIGHT DIAGNOSTICS™ Rabies DFA 5100 Reagent (Millipore, Billerica, Massachusetts, USA)[1,9].

## RESULTS

### *The Immune Effect of Synthetic Peptide on BALB/c Mice*

Indirect ELISA was used to detect anti-peptide antibody titer in serum samples. Both BALB/c mice exhibited low background anti-peptide antibody before immunization, which was acceptable for further immunization with the conjugated peptide (Table 1). After three booster immunizations, the anti-peptide antibody titer in each mouse serum sample was about 1:81000 (Table 1). One of the two mice was used to prepare hybridoma cell lines.

**Table 1.** OD Values of Two Balb/c Mice Sera Samples before and after Immunization as Detected by Indirect ELISA

| Dilution | Before Immunization | | After Immunization | |
|---|---|---|---|---|
| | $OD_{mouse1}$ | $OD_{mouse2}$ | $OD_{mouse1}$ | $OD_{mouse2}$ |
| 1:1000 | 0.136 | 0.137 | 4.003 | 4.020 |
| 1:3000 | 0.133 | 0.135 | 3.872 | 3.880 |
| 1:9000 | 0.110 | 0.116 | 3.663 | 3.680 |
| 1:27000 | 0.097 | 0.090 | 2.920 | 2.933 |
| 1:81000 | 0.083 | 0.086 | 0.893 | 0.880 |
| 1:243000 | 0.071 | 0.075 | 0.126 | 0.131 |
| 1:729000 | 0.069 | 0.064 | 0.096 | 0.092 |
| Blank control | 0.041 | 0.039 | 0.049 | 0.050 |
| Negative control | 0.070 | 0.069 | 0.073 | 0.075 |

### *Screening of Hybridoma Cell Lines*

Splenocytes were harvested and fused with SP 2/0 myeloma cells. Hybridoma supernatants were screened using indirect ELISA and IFA. Sixteen positive clones were identified by indirect ELISA. Of these 16 clones, two tested positive by IFA (RVNP-mAb1-CL and RVNP-mAb2-CL), although RVNP-mAb2-CL was weakly positive (Figure 1). Based on the anti-IgG type of secondary antibody, RVNP-mAb1 and RVNP-mAb2 were of IgG type of immunoglobulin.

Two BALB/c mice were intraperitoneally inoculated with RVNP-mAb1-CL and RVNP-mAb2-CL hybridomas, respectively. The ascites were assessed using IFA in serial dilution. The lowest dilution showing a stable fluorescent signal was 1:200. The fluorescent signal of RVNP-mAb1 was stronger than that of RVNP-mAb2 (Figure 2), indicating a higher concentration of antibody in RVNP-mAb1-CL ascites. RVNP-mAb1-CL was therefore selected for large-scale production of RVNP-mAb1.

### *Purification and Labeling of RVNP-mAb1*

Ten BALB/c mice were intraperitoneally inoculated with RVNP-mAb1-CL hybridoma cells, and a total volume of 50 mL of ascites was harvested and pooled. RVNP-mAb1 was purified by DEAE-Sephadex A-50 column chromatography. SDS-PAGE of purified RVNP-mAb1 showed a single protein band of approximately 50 kDa (Figure 3). The concentration of purified RVNP-mAb1 was 20 mg/mL in a total volume of 40 mL. Purified RVNP-mAb1 was labeled

with FITC, and unbound antibody and FITC were removed. The concentration of FITC-labeled RVNP-mAb1 was 16 mg/mL in a total volume of 35 mL. The yield of labeled antibody was 70%. FITC-labeled RVNP-mAb1 was diluted to 1 mg/mL and distributed in 1 mL/tube for further use.

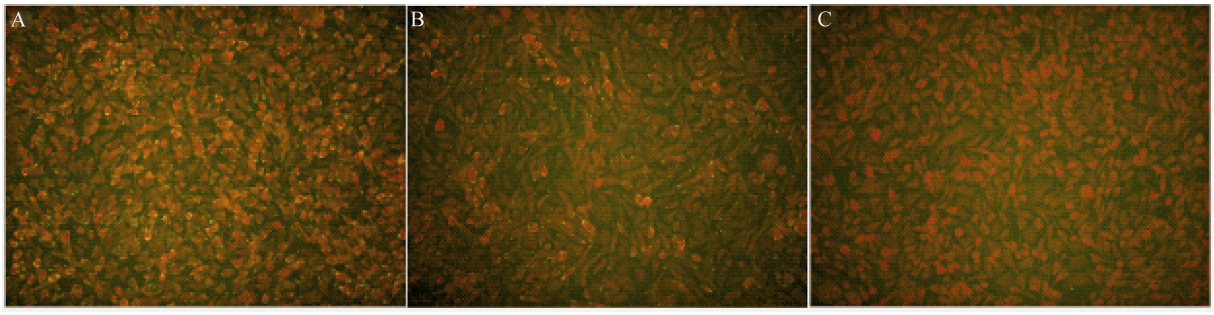


**Figure 1.** IFA using hybridoma supernatants (200×magnification). A, RVNP-mAb1; B, RVNP-mAb2; C, Negative control.

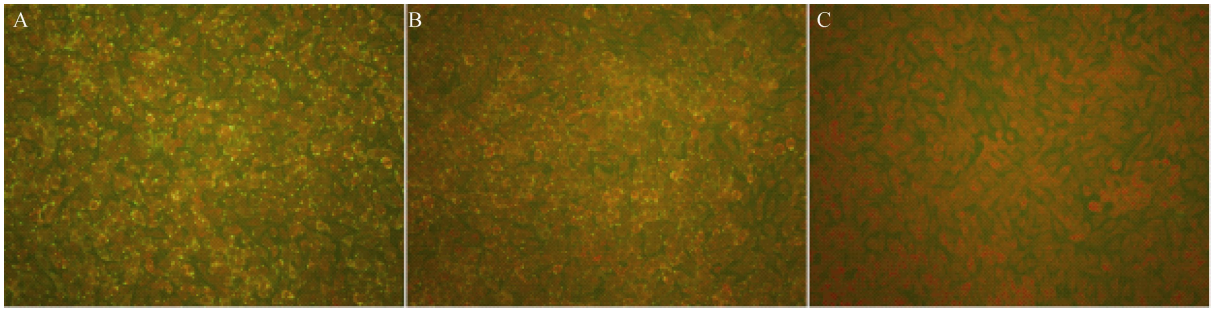


**Figure 2.** IFA using ascites of BALB/c mice inoculated with positive clones at a 1:200 dilution (200×magnification). A, RVNP-mAb1; B, RVNP-mAb2; C, Negative control.

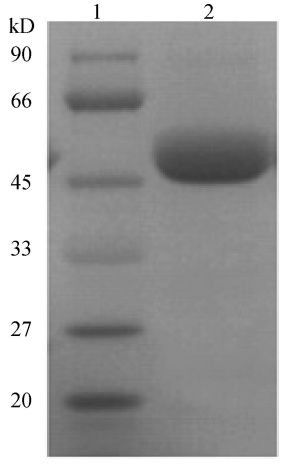


**Figure 3.** The protein profile of purified RVNP-mAb1 in 10% SDS-PAGE. 1. Protein marker; 2. Purified RVNP-mAb1.

### *Preliminary Assessment of the Efficacy of FITC-labeled RVNP-mAb1*

Four canine brain tissue samples diagnosed by LIGHT DIAGNOSTICS™ Rabies DFA 5100 Reagent were used to assess the efficacy of RVNP-mAb1 for detection of *Rabies virus*. FITC-labeled RVNP-mAb1 was diluted from 1:50 to 1:500 by increments of 50 and used in FAT. FITC-labeled RVNP-mAb1 effectively recognized *Rabies virus* in canine brain tissue

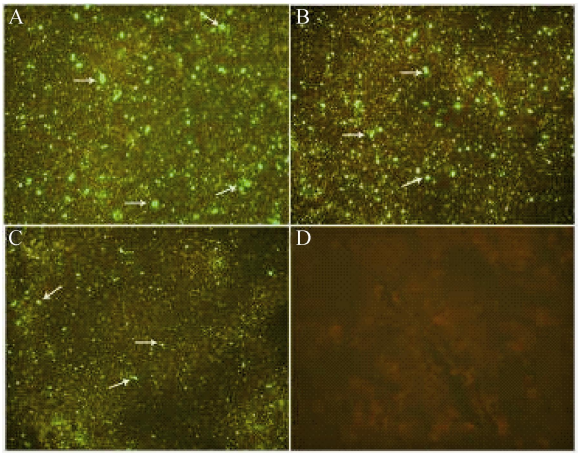


**Figure 4.** FAT for *Rabies virus*-positive canine brain tissue samples using FITC-labeled RVNP-mAb1 (200×magnification). A-C, Positive canine brain tissue (white arrows indicate positive signal); D, Normal canine brain tissue.

samples at serial dilutions up to 1:200. These qualitative results obtained by FITC-labeled RVNP-mAb1 are consistent with those diagnosed by LIGHT DIAGNOSTICS™ Rabies DFA 5100 Reagent (Figure 4). The working concentration for each antibody reagent was equivalent to 5 μg/mL. Similarly, the quantitative results of RFFIT from 200 human serum samples assayed by both antibody reagents were highly correlated[9].

## DISCUSSION

As a standard rabies laboratory diagnostic method recommended by WHO, FAT is widely accepted in rabies laboratories around the world[1]. High-quality anti-*Rabies virus* nucleoprotein monoclonal antibodies are critical components of FAT. However, anti-*Rabies virus* nucleoprotein monoclonal antibodies as commercial diagnostic reagents are expensive and scarce. Few researchers have applied hybridoma technology to prepare anti-*Rabies virus* nucleoprotein monoclonal antibodies with satisfactory results for rabies laboratory tests[3,7]. These anti-*Rabies virus* nucleoprotein monoclonal antibodies were focused primarily on conserved antigenic epitopes[7-8]. When *Rabies virus* nucleoprotein is used as an immunogen, screening hybridoma cell lines is labor intensive[14]. The specific epitope-rich region has been used as an immunogen, which simplified the selection of hybridoma cell lines for desired epitopes[15]. The synthetic peptide containing a newly discovered conserved linear epitope of *Rabies virus* nucleoprotein was first used to prepare monoclonal antibodies in the present study.

Rabies is mainly a danger to people in rural areas of developing and less-developed countries in Asia and Africa. These countries have the most urgent demand for practical rabies laboratory diagnostic techniques and reagents[16-17]. Adapting general laboratory conditions are important factors to improving the rate of rabies diagnosis in these countries[8]. Self-prepared anti-*Rabies virus* nucleoprotein monoclonal antibodies have been used for rabies diagnosis by FAT, sandwich ELISA-WELYSSA, and direct rapid immunohistochemical tests (dRIT)[7-8]. RVNP-mAb1 was prepared using mature methods[12] and produced in large-scale. Initial application of RVNP-mAb1 showed its potential of being a laboratory diagnostic reagent for rabies. The widespread use of RVNP-mAb1 has improved the rate of rabies diagnosis independent of commercial reagents.

RVNP-mAb1 is a monoclonal antibody against a newly discovered conserved linear epitope of *Rabies virus* nucleoprotein predicted by bioinformatic analysis[18-19]. It was necessary to verify the ability of RVNP-mAb1 to recognize many strains of *Rabies virus*. However, limited *Rabies virus* was detected. To systematically evaluate RVNP-mAb1, more *Rabies virus*-positive canine brain tissue samples should be evaluated using standard statistical guidelines[20]. *Rabies virus* is one species in the *lyssavirus* genus[21]. It will be interesting to evaluate the capability of RVNP-mAb1 to recognize the other 10 species in *lyssavirus* genus[20-21]. Since only a few top rabies laboratories in the world are able to test for multiple species of *lyssavirus*, international cooperation will be necessary to complete this study. In addition, preparation of monoclonal antibodies against other potential antigenic epitopes of *Rabies virus* nucleoprotein may contribute to the establishment of an anti-*Rabies virus* nucleoprotein monoclonal antibody bank[18,20]. Such a bank would be useful for identifying multiple *Rabies* viruses with antigenic differences[20].

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