Preparation and Identification of Anti-rabies Virus Monoclonal Antibodies[*]

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Abstract: To provide a foundation for the development of rapid and specific methods for the diagnosis of rabies virus infection, anti-rabies virus monoclonal antibodies were prepared and rabies virus nucleoprotein and human rabies virus vaccine strain (PV strain) were used as immunogens to immunize 6-8 week old female BALB/c mice. Spleen cells and SP2/0 myeloma cells were fused according to conventional methods: the monoclonal cell strains obtained were selected using the indirect immunofluorescence test; this was followed by preparation of monoclonal antibody ascitic fluid; and finally, systematic identification of subclass, specificity and sensitivity was carried out. Two high potency and specific monoclonal antibodies against rabies virus were obtained and named 3B12 and 4A12, with ascitic fluid titers of 1:8000 and 1:10000, respectively. Both belonged to the IgG2a subclass. These strains secrete potent, stable and specific anti-rabies virus monoclonal antibodies, which makes them well suited for the development of rabies diagnosis reagents.

Key words: Rabies virus; Monoclonal antibodies; Specificity; Detection

Monoclonal antibodies against rabies virus have been widely used in the diagnosis and immunological analysis of rabies[7,13]. Human monoclonal antibodies to rabies G protein are also expected to be used as a replacement for rabies immune globulin(RIG)[4,10] in the post-exposure treatment of rabies. Two techniques are recommended by WHO and used world wide. These are the gold standard fluorescent antibody (FA) technique, a rapid and sensitive method for diagnosing rabies infection in animals; and the rapid fluorescent focus inhibition test (RFFIT) for the evaluation of neutralizing antibodies of rabies virus (RV). Both

these techniques need fluorescent-labeled high potency and specific antibodies to RV antigens. However, this detection reagent is not yet approved for commercial manufacture in China, while the imported products are expensive and therefore are not readily available. This mitigates against the use of these techniques as standards. Thus, the preparation of potent and specific monoclonal antibodies against rabies virus will contribute significantly to development of techniques for laboratory diagnosis of rabies. Since these will have local independent property rights it will facilitate rabies surveillance in China.

In 1978, Wiktor[11] reported the preparation of rabies virus monoclonal antibodies. Since then, rabies virus monoclonal antibody (McAb) technology has been more and more widely used in basic research, and the testing and diagnosis of rabies. In this study, anti-RV monoclonal antibodies were prepared using a human RV vaccine strain and a baculovirus-expressed RV nucleoprotein as antigens.

MATERIALS AND METHODS

Cells and animals for immunization

Mouse SP2/0 myeloma cell strains were provided by the Xinjiang Uygur Autonomous Region Center for Disease Control and Prevention; BSR cells were kept in our laboratory. Animals used for immunization were 6-8 weeks old female BABL/C mice.

Antigens for immunization

Concentrated solutions of human rabies virus vaccine PV strain were provided by the ChengDa Bio Co., Ltd. Rabies virus nucleoproteins (CVS-11 strain) were expressed and purified by a baculovirus eukaryotic expression system [3] in our laboratory.

Reagents and instruments

PEG4000 (Sigma Corporation), FITC labeled goat anti-mouse IgG and murine monoclonal antibodies subtype identification kit ISO. 2 (Sigma, USA); HT and HAT medium (Invitrogen, USA); nProtein A Sepharose 4 Fast Flow (Pharmacia Corporation, USA); anti-RV nucleoprotein fluorescent antibody (Millipore Corporation); Freund's complete and incomplete adjuvant (Sigma, USA).

Processing of antigens for immunization

Antigens used for immunization were rabies virus nucleoproteins (CVS-11 strain) and concentrated solution of human rabies virus vaccine PV strain. The concentrations of two antigen suspensions were in the range of 1-1.5 mg/mL after measurement by UV-spectrophotometry, they were then diluted with PBS to the required concentrations (Table 1).

Animal immunization

Ten 6-8 week-old female BABL/C mice were divided into two groups. Immunizations were conducted on d 1, 15, 29 and 43 respectively according to methods described elsewhere [9], four times in total. The first three injections were on both sides of the subcutaneous tissue of the neck, back and into the peritoneal cavity. The final immunization was into the peritoneal cavity, the other details are shown in Table 1. After immunization, blood was collected from the caudal vein and specific anti-RV antibody

Table 1. Immunization process of rabies virus (CVS-11 strain) nucleoprotein and PV strain antigens in mice

Immunization number	Period /d	Dose /μg	Adjuvant
1	1	150	Freund's complete adjuvant
2	15	75	Freund's incomplete adjuvant
3	29	75	Freund's incomplete adjuvant
4	43	75	None

levels in serum were detected using indirect immunofluorescence to evaluate immune efficacy.

Preparation of cells

Preparation of myeloma cells: SP2/0 myeloma cells were cultured in RMPI1640 medium containing 10% serum at 37℃ and 5% CO_2, and passaged once every two days. SP2/0 cells from the logarithmic phase were selected for the fusion with spleen cells.

Preparation of feeder cells: One healthy Balb/c mouse was killed by cervical dislocation the day before cell fusion, 5 mL 1640 medium (containing 20% serum) was injected into the peritoneal cavity of the mouse, then the medium was pumped out after gentle massage on the mouse peritoneal cavity and transferred into flasks. The sufficient medium was added and mixed with the feeder cells, 200 μL/well was distributed into 96-well plates and incubated at 37℃, 5% CO_2 overnight.

Fusion of spleen cells with SP2/0 cells

A booster immunization was conducted on the immunized mice. Three days later, spleen cells of the mice which showed better immune efficacy were selected for the fusion according to the methods described[1,2,8], the spleen cells were mixed with SP2/0 cells at a 5-10:1 ratio. After centrifugation, 1 mL 50% PEG4000 was added into the mixed cells drop by drop over 1 min and after 90 sec serum-free RMPI-1640 medium was added to stop the reaction. After centrifugation, the fusion cells were resuspended using RMPI-1640 selective medium containing 20% serum (FBS) and 1% HAT, and 100 μL/well of it was added into 96-well plates already loaded with the feeder cells. The plates were then incubated at 37℃, 5% CO_2.

Screening of positive hybridoma

Preparation of CVS-11 RV antigen plate: BSR cells were infected with rabies virus CVS-11 strain then paved in a monolayer in 96-well plates; after 24 h of incubation, each infected cell plate was fixed with acetone and then used for screening of the hybridoma.

Screening of the hybridoma: The fusion result was checked every day. On day 8, cell supernatant of the wells in 96-well plates was detected using CVS-11 RV antigen plate by indirect fluorescent antibody (IFA) technique:50 μL supernatant of the selected hybridoma was added into the wells of CVS-11 RV antigen plate and incubated at 37℃ for 30 min, then FITC-goat anti-mouse IgG was added. After 30 min at 37℃, 60% glycerol was added, observation was conducted through the fluorescence microscope. The positive results showed as specific yellow-green fluorescent granules against the red colored cell background.

Cloning and cell strain construction of the positive hybridoma

After the screening of positive hybridoma, Double dilution could be conducted directly for single cell cloning. The positive cloned wells were selected and cloning continued, this was repeated for the second cloning, after which the positive cells were used for the construction of cell strains.

Antibody secretion of cell strains was detected every other generation after the construction of cellstrains. Cell strains which showed four consecutive positive detection results were selected, the hybridoma was gradually expanded from 48-well plates to 24-well plates and then to 6-well plates and finally a flask.

Preparation of anti-rabies virus monoclonal antibody ascitic fluid and titration

After being expanded in the flask, the monoclonal cell strains then expanded *in vitro* using mouse

intraperitoneal amplification. 0.5 mL cell suspension containing 1.0×10^{6} monoclonal aitibody cells was injected intraperitoneally into multiparous BABL/C mice; each monoclonal cell strain was injected into 5 mice. Ascitic fluid was collected after 7-10 days. Ascitic fluid obtained was purified using SPA affinity chromatography; concentration of purified monoclonal antibodies was detected using UV spectrophotometry. The purification result was checked by SDS-PAGE.

Ascitic fluid titration: Using IFA technique, the ascitic fluid obtained was taken as the primary antibody source for double dilution and 10-fold serial dilution respectively starting from 1:1000. The final concentrations for the two dilutions were 10000 and 10^{10} respectively. Observation and analysis were conducted by inverted fluorescence microscope.

Anti-rabies virus neutralizing monoclonal antibodies titer

Using the RFFIT method[12], the viruses obtained with the optimum infection level and monoclonal antibodies supernatant were neutralized. The greatest dilution of monoclonal antibody supernatant able to neutralize 50% of the virus was calculated and antibody neutralizing titer of monoclonal antibody supernatant was compared with standard serum with known titer, thus demonstrating the ability of the obtained monoclonal antibodies to neutralize rabies virus.

Identification of anti-rabies virus monoclonal antibody subclass

ELISA plates were coated with different subclasses of murine monoclonal antibodies. Purified ascitic fluid was taken for primary antibodies, the types of monoclonal antibodies being detected using ELISA capture according to the ISO.2 (Sigma, USA) manual.

RESULTS

Immunization assessment of animals

Blood from the tails of immunized mice was used for the immunization assessment and the serum antibody titer was detected using IFA technique. The results showed that the highest antibody titers of 10 immunized mice reached 1:6000, indicating effective immunization.

Results of fusion and clone screening

The fusion rate was higher than 80%. Clone screening was carried out twice. Results showed screening reduced the number of positive clone wells, because positive hybridoma can lose the ability to secrete antibodies due to factors such as damage during the passage process. Eventually, positive hybridoma that showed high-titer and specific secretions of anti-rabies virus were selected for construction of cell strains. Details are shown in Table 2.

Stability testing of positive clone cells

Table 2. Fusion and screening results of mice immunized with rabies virus nucleoprotein and concentrated vaccine for human use

	Expressed nucleoprotein immunization	Human vaccine PV strain
Fusion proportion	92.6%	83.9%
Number of positive cells after first screening	42	39
Number of positive cells after second screening	6	7
Number of positive cells after third screening	1	1
Positive proportion of single-well cloning after the first cloning	40.6%	43.8%
Positive proportion of single-well cloning after the second cloning	100%	100%

Table 3. Detection of antibody-secretion of hybridoma cell strains after construction of cell strains

Cell strains	1st generation	3rd generation	5th generation	11th generation	Detection after resuscitation
3B12	1:800	1:800	1:800	1:800	1:800
4A12	1:900	1:900	1:900	1:900	1:900

The passage number after the construction of cell strains was taken as the first generation. The stability of the first eleven generations of cells was detected at every other generation, as shown in Table 3. The antibody titer of the supernatant of monoclonal cell strains was maintained at 1:800-900, showing a good stability and high specificity. The indirect FA results of the monoclonal antibody supernatant are shown in Fig. 1. The presence of specific yellow-green fluorescent granules indicates viral antigens are in the monoclonal antibody supernatant. This demonstrates that the monoclonal antibodies obtained showed good specificity, sensitivity and stability. These granules do not appear in the negative control.
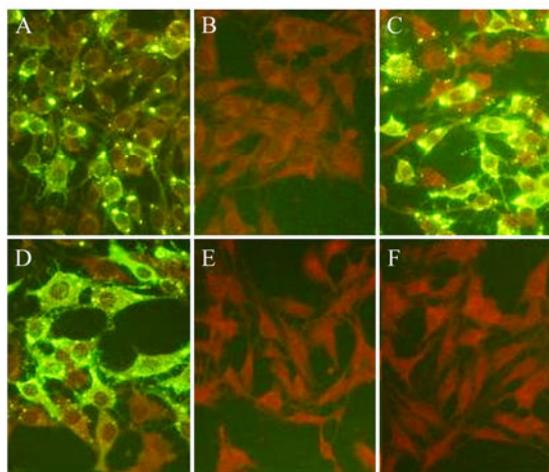

Fig. 1. IFA results of the monoclonal antibody cell supernatant under 200× inverted fluorescence microscope. A, 3B12 strain monoclonal antibody; B, 3B12 blank cell control; C, 4A12 strain monoclonal antibody; D, anti-RV nucleoprotein monoclonal antibody from the Millipore Corporation; E, 4A12 blank cell control; F, blank negative control.

Purification and identification of monoclonal ascitic fluid

The two monoclonal antibody cell strains, 3B12 and 4A12 were detected for the neutralization titer through RFFIT, the results are shown in Table 4.

The titers of obtained ascitic fluid for the two strains, determined by indirect immunofluorescence, were 1:10000 and 1:8000 respectively. UV spectro-photometric analysis showed the concentration of monoclonal antibodies of purified ascitic fluid were 1.06 mg/mL and 0.80 mg/mL respectively. SDS-PAGE analysis after purification by electrophoresis (Fig. 2) shows that, compared to the vague band of unpurified samples, the purified monoclonal antibodies from the ascitic fluid present two clear bands near 25 KD and 50 KD, consistent with the expected molecular size of the IgG light chain and heavy chain.

Identification result of antibody subclasses

Sub-class identification was conducted using ELISA capture with a monoclonal antibodies subclass identification kit (Sigma), and both monoclonal antibodies obtained were determined as IgG2a monoclonal antibodies.

Table 4. Neutralization titer of 3B12 and 4A12 detected by RFFIT.

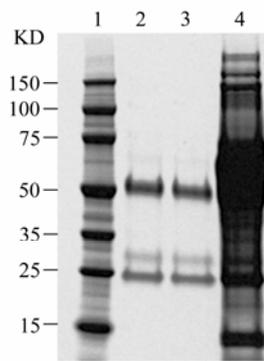| Sequence number	Sample	Neutralization titer （IU/mL）
1	4A12 cell supernatant	4.06
2	3B12 cell supernatant	3.28
3	3B12 ascitic fluid	9.25
4	4A12 ascitic fluid	4.29

Fig. 2. Analysis result of purified ascitic fluid by SDS-PAGE Electrophoresis. 1, Marker; 2 and 3 are the purified monoclonal antibodies: 3B12 and 4A12; 4, unpurified ascitic fluid.

DISCUSSION

In this study, we report the production of anti-rabies virus monoclonal antibodies as the first step in the development of a rapid and specific reagent for the diagnosis of rabies virus infection. In the process of developing this kind of reagent, we observed a number of important considerations necessary for an effective and efficient technique. The experimental immunogens used in this study were an inactivated rabies virus vaccine strain (PV) for human use and an expressed nucleoprotein of rabies virus. They were both diluted in the proportion of 1:10 after concentration for immunization and since they retained some cellular components, the generation of anti-cellular monoclonal antibodies was inevitable. To minimize the false positive results, a negative cell control was used during identification. The injection method is conducive to generating large numbers of spleen B-lymphocytes. The results showed that the spleens of mice after full-course immunization were double that of normal mice, with the number of spleen cells up to 2.0×10^8.

In this study, dilution was conducted in advance of fusion using twenty 96-well plates with, in principle,

only 1-2 cells in each well. The results showed that the proportions of single cells were 21.4% and 19%, respectively, which was different from the traditional fusion step conducted directly on the fused single cells, eliminating the need to clone a large number of the fused cells. The number of cloning cycles in the actual experimental process could be reduced to one or two.

To increase the survival rate of hybridoma, feeder cells were prepared l-2 d before fusion. Peritoneal macrophages of syngenic mice were usually selected as feeder cells. Generally, feeder cells prepared from Balb/c mice weighing about 18g can pave three to four 96-well cell culture plates. The activity and the pollution status of cells were observed on the second day; cells which show a poor activity, no adherence or have been polluted cannot be used. The loss of certain chromosomes during the amplification can cause the instability of fusion cells[6]. That is why the number of positive hybridoma decreased with time, as shown in the result. Timely cloning is a more important consideration for efficient generation of monoclonal antibodies because in the same well, the growth ability of cells which do not secrete antibodies will be greater than those secreting antibodies [4].

The titer of the neutralizing antibodies obtained was detected using the RFFIT and the results showed that the generated monoclonal antibodies had the ability to neutralize rabies virus. The neutralizing activity needed to be further validated and the details are shown in Table 4.

There are many ways to purify monoclonal antibodies[14]: including ammonium sulfate precipitation, octanoic acid precipitation and affinity chromatography. Of these, ammonium sulfate precipitation shows a higher purified rate of ascitic fluid but a lower

recovery rate, and it has a significant impact on the immune activity of IgG[5], while octanoic acid precipitation achieves the opposite result. Therefore, in this experiment, using affinity chromatography, our experimental results verified that this purification method was efficient, with SDS-PAGE bands showing clearer bands present near 50 KD and 25 KD. One corresponds to the IgG heavy chain, at about 50 KD, and the other corresponds to the IgG light chain, at about 25 KD; there are many more protein bands in unpurified samples, indicating that the vast majority of impure protein is removed after the purification of ascitic fluid.

Presently, rabies is an important zoonotic disease seriously threatening public health. Rapid and effective diagnoses as well as proper preventive measures are particularly important. Monoclonal antibodies prepared in this experiment were identified as anti-rabies virus monoclonal antibodies and have an important application value. Labeling these monoclonal antibodies and developing corresponding detection techniques will be the next steps of to allow their use in the near future for the diagnosis of rabies virus infection and control of rabies vaccine quality.

Acknowledgment

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