Immune-Tolerizing Procedure for Preparation of Monoclonal Antibodies Against Glycoprotein E of Pseudorabies Virus

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The glycoprotein E (gE) of pseudorabies virus (PRV) is known to be an important marker protein in the control and eradication of Aujeszky's disease. In this study, BALB/c mice were immunized with gE-deleted PRV as tolerogen and with wild-type PRV as immunogen. The spleen cells from the immunized mice were then fused with the myeloma cell line Sp2/0. Two hybridoma cell lines that could stably secrete the monoclonal antibody (MAb) against gE were achieved by using indirect ELISA screening and subcloning three times; they were named 1D2 and 2B2. Indirect immunofluorescence assay (IFA) revealed that the MAbs were specifically against gE of PRV. MAbs 1D2 and 2B2 were subgroup IgG1. The MAbs obtained in this study provide useful tools for the development of differential diagnostic methods for PRV.

Introduction

PSEUDORABIES VIRUS (PRV), also known as Aujeszky's disease virus, is a herpesvirus primarily found in swine. The virus can establish infection in most mammals, with the exception of humans.⁽¹⁾ In young piglets, PRV infection is often fatal, the animals dying from central nervous system disorders. Older pigs generally carry the virus in a latent form for their entire life. In pregnant sows, PRV infection normally leads to reproductive failure.⁽²⁾

Vaccination is one of the most effective ways to control pseudorabies. In the United States and some other countries, pseudorabies has been successfully eradicated by using gene-deleted vaccines and the accompanying diagnostic test, which could distinguish infected from vaccinated animals.^(3–5) At present, the most widely used vaccines against PRV in China are gE gene-deleted vaccines that failed to produce the non-essential protein gE.⁽⁶⁾ Thus, the generation of MAbs against gE and development of specific methods for detecting gE antigen or serum antibodies against gE will contribute to PRV eradication programs in this country.

The gE of PRV belongs to a typical type I transmembrane protein with four conformational epitopes.⁽⁷⁾ It is hard to obtain complete gE by expressing full length gE gene in heterologous cells. It is therefore preferable to prepare high affinity monoclonal antibodies against PRV by using inactivated whole virus as immunogen. However, it will reduce screening efficiency of gE-specific MAb, since there are many different glycoprotein proteins on the surface of PRV

besides gE, such as glycoproteins B and C. Therefore, an immune-tolerizing procedure was employed to generate gE-specific MAbs in the present study.⁽⁸⁾ Wild-type PRV and gE-deleted PRV were used as immunogen and tolerogen, respectively. Finally, two hybridoma cell lines secreting MAbs against gE were selected and characterized.

Materials and Methods

Chemicals and reagents

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) was purchased from Southern Biotechnology Associates (Birmingham, AL). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H+L) was obtained from Zymed Laboratories (San Francisco, CA). RPMI-1640 with L-glutamine was obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co. (Hangzhou, China). Freund's adjuvant and cyclophosphamide (CY) were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of tolerogen and immunogen

gE-deleted PRV strain Ea/gE⁻ and wild-type PRV strain Ea were used as tolerogen and immunogen, respectively. For preparation of pseudorabies virus, PK-15 cell monolayers were incubated with viral solutions and cultured at 37°C. When typical cytopathic effects (CPE) appeared, the cell cultures were collected. After freezing and thawing three times,

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virus cultures were inactivated by 0.2% formaldehyde and centrifuged at 3000g for 15 min to remove cell debris. The virus in the supernatant was then purified through zonal sucrose gradient centrifugation.⁽⁹⁾ The virus was mainly allocated between 35~45% sucrose gradients.

Mice and immunization

BALB/c mice (female) were purchased from the Centre for Disease Control and Prevention (Hubei Province, China). Mice were maintained under standard animal housing conditions, with a temperature of $22 \pm 1^\circ\text{C}$, a regular 12-h light/12-h dark cycle, and free access to food and water. The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals established by the Centre for Disease Control and Prevention (Hubei Province). The immunization was performed as described previously with some modifications.⁽¹⁰⁾ Firstly, six-week-old BALB/c mice were intraperitoneally injected with 70 μg gE-deleted PRV (tolerogen). Then, the mice were intraperitoneally injected with 100 mg/kg CY at 15 min, 24 h, and 48 h after the injection of tolerogen. Two weeks later, the tolerogen injection and CY treatment procedures were repeated once. After being treated with CY, the mice were immunized with 70 μg wild-type PRV (immunogen) three times with a two-week interval. Complete Freund's adjuvant and incomplete Freund's adjuvant (Sigma-Aldrich) were used in the first immunization with immunogen and the subsequent two booster shots, respectively. A final immunization with 70 μg wild-type PRV was given intravenously 3 days before euthanasia.

Cell fusion

Cell preparation and fusion were performed as described previously.⁽¹¹⁾ Briefly, spleens from BALB/c mice immunized with wild-type PRV were harvested and splenocytes were prepared in Hank's salt solution (Invitrogen). For hybridoma preparation, splenocytes were fused with mouse myeloma cells SP2/0 at a ratio of 10:1 in RPMI-1640 medium at 37°C . The splenocytes and myeloma cell mixture were centrifuged and resuspended in 0.8 mL 50% PEG1450 solution (Sigma-Aldrich) over 1 min, followed by serial addition of 3 mL RPMI-1640 over 3 min and 10 mL RPMI-1640 over 1 min, with gentle stirring. Fused cells were centrifuged at 1000 rpm for 5 min, and resuspended in 200 mL of RPMI-1640 medium, which contained hypoxanthine, aminopterin, and thymidine (Sigma-Aldrich) and 10% fetal bovine serum. Fused cells were then plated at 200 μL /well into 96-well cell culture plates (Falcon, BD Biosciences, Auckland, New Zealand). Cells were incubated at 37°C , with 5% CO_2 for 14 days. Supernatant of positive wells were tested for antibody production by ELISA screening. Positive clones were cloned three times by limiting dilution using spleen cells as feeder cells.

ELISA screening

Wild-type PRV was used to establish indirect ELISA for screening MAbs. 96-well plates were coated with 2 $\mu\text{g}/\text{mL}$ of PRV diluted in coating buffer (50 mM sodium carbonate buffer, pH 9.6) at 4°C overnight. The coated plates were washed three times with PBST (PBS with 0.05% Tween-20) and blocked for 1 h with blocking buffer (5% skimmed milk in

PBST), then washed three times with PBST. Cell culture supernatants (100 μL aliquots) were added and incubated at 37°C for 30 min. After four washes, 100 μL of HRP-conjugated goat anti-mouse IgG, diluted to 1:5000 in PBST, was added to each well, and the plates were incubated in 37°C for 30 min. Next, plates were washed five times with PBST, and ELISAs were developed using 100 μL 3, 3',5, 5'-tetramethylbenzidine (TMB) color development solution (Biotime Institute of Biotechnology, Haimen, Jiangsu, China). The catalytic reaction was stopped by 50 μL 0.025% hydrofluoric acid and read at 630 nm. In the ELISA screening, plates coated with the same molar concentration of gE-deleted PRV served as a negative control.

MAb production and purification

To produce anti-gE MAbs, female BALB/c mice were injected intraperitoneally with 0.5 mL of incomplete Freund's adjuvant, then intraperitoneally injected with the positive hybridoma cells (2×10^6 cells/mL) suspended in 0.5 mL RPMI 1640 medium after 10 days. Ascitic fluid was collected 10–14 days after the injection. Purification of MAb was performed according to the modified caprylic acid ammonium sulphate precipitation (CAASP) method as described previously.⁽¹²⁾ The purified MAbs were analyzed by SDS-PAGE.

Determination of MAb specificity

The specificity of the MAbs was evaluated by the ELISA methods described above and indirect immunofluorescence assay (IFA). For IFA, PK-15 cells in wells of tissue culture plates were infected with wild-type PRV. When typical CPE appeared, PK-15 cells were fixed by using 70% cold acetone.

FIG. 1. SDS-PAGE analysis of the purified MAbs. M, protein marker (Fermentas, Vilnius, Lithuania); lane 1, purified MAb 1D2; lane 2, purified MAb 2B2.

FIG. 2. Immunofluorescence analysis of MAbs. A1, wild-type PRV-infected PK-15 cells incubated with 1D2; A2, gE-deleted PRV-infected PK-15 cells incubated with 1D2; B1, wild-type PRV-infected PK-15 cells incubated with 2B2; B2, gE-deleted PRV-infected PK-15 cells incubated with 2B2. (400 $\times$)

After fixation, the cells were washed thrice with PBS. Then cells were incubated with different MAbs at 37°C for 30 min, followed by three washes with PBS. Cells were then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H+L) for another 30 min away from light, followed by three washes with PBS. Finally, the cell samples were examined under a fluorescence microscope (Olympus, Tokyo, Japan). PK-15 cells infected with gE-deleted PRV were used as negative control.

Results

Mice immunization

Following three booster immunizations, mice sera antibodies against tolerogen and immunogen were evaluated by

indirect ELISA, respectively. Results indicated that all the immunized mice had high antibody titers (ranging from 1:6400 to 1:12,800) against immunogen after three booster immunizations, while the antibody titers against tolerogen were below 1:3200.

Preparation of hybridoma cell lines, isotype analysis, and MAb production

Three days after the last booster, the mouse with the highest antibody titer against immunogen was splenectomized and the spleen cells were fused with SP2/0 myeloma cells. Supernatants from the positive wells were detected by indirect ELISA. Eventually, two hybridomas secreting the MAbs against immunogen were selected following limiting dilution method, designated as 1B2 and 2D2, respectively. Isotyping

FIG. 3. MAb titers against gE. MAbs were collected from the supernatants of hybridomas and serially diluted, then added into the immunogen-coated 96-well ELISA plates, respectively. Supernatant from SP2/0 cells served as negative control. The ELISA assays were performed at least in triplicate. Data represent mean values of three replicates.

results indicated that the two MAbs were of subclass IgG1. Ascites including MAbs were purified by CAASP, and the concentrations of the MAbs were ~1.1 mg/mL (Fig. 1, lanes 1, 2).

Specificity analysis of MAbs

The specificity of the MAbs obtained here was further investigated by IFA and ELISA. Under the fluorescent microscope, PK-15 cells infected by wild-type PRV showed a strong green fluorescence (Fig. 2, A1, B1). No evident fluorescence was observed in the cells infected by gE-deleted PRV (Fig. 2, A2, B2). The results indicated that two MAbs did not react with gE-deleted PRV, but they were directed against the wild-type PRV. The affinity of MAbs to gE were determined by ELISA using serial dilutions of MAbs. The results indicated that these MAbs had high titers with wild-type PRV, and the supernatants from SP2/0 myeloma cell culture were negative in the ELISA analysis (Fig. 3).

Discussion

Pseudorabies is a viral disease in swine that is endemic in most parts of the world. It is caused by the pseudorabies virus. To control and eradicate PRV, gE-negative marker vaccines are used in most countries.^(13–15) The use of gE-negative vaccines makes it possible to distinguish vaccinated from infected pigs by detection of gE or gE antibodies. Because gE MAbs can be used in the differential diagnosis of pseudorabies disease, it is critical to obtain gE MAbs for the development of testing methods.

Recombinant gE protein expressed in *E. coli* was used as immunogen to generate gE-specific MAbs. However, the specificity and affinity of MAbs to the native gE protein from PRV were too low to be used for further study. This might be due to the lack of natural conformation and glycosylation modification of recombinant antigen. The preferable way to prepare gE MAbs remains using PRV as immunogen. But the conventional immunization method might elicit low levels of gE-specific antibodies in the immunized mice. Thus, in this study, an immune-tolerizing method (also called *subtractive immunization*⁽¹⁰⁾) was performed for MAb preparation. In the immune-tolerizing procedure, CY could induce immune tolerance to gE-deleted PRV. After three immunizations with wild-type PRV, mice sera were tested by ELISA. The results revealed that the mice had high antibody titers against wild-type PRV but low antibody titers against gE-deleted PRV. These results indicate that the mouse immune system mainly produced lymphocytes that secreted antibodies against gE. The immune strategy not only improved production of gE-specific antibodies, but also reduced non-specific reaction in the screening processes.

Considering the fact that the native conformation of gE might lose in the treatment process of Western blot analysis, IFA was performed to further evaluate the specificity of the MAbs. IFA results showed that the MAbs bound specifically to PRV containing gE but did not react with gE-deleted PRV. This illustrated that the MAbs could recognize natural gE protein and have no cross-reaction with other structure proteins of PRV or PK-15 cells. Affinity measurement also indicated that the MAbs had high titers with gE. Of course, specific epitopes recognized by the MAbs will need to be further analyzed.

In summary, this study provides a quick and high-performance preparation of MAbs against gE of PRV. The MAbs generated here will be useful for the development of diagnostic methods and further functional analyses of gE protein.

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Author Disclosure Statement

The authors have no financial interests to declare.

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