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RESEARCH ARTICLE

Development and Characterization of a Panel of Monoclonal Antibodies Against Pseudorabies Virus gE/gI Complex

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ABSTRACT

gE/gI complex of pseudorabies virus was expressed in Baculovirus expression system. Female BALB/c mice were immunized with the purified gE/gI complex and 22 monoclonal antibodies (mAbs) were achieved by hybridoma technique. A panel of monoclonal antibody was used in indirect immune fluorescence assay(IFA), immunoperoxidase monolayer assay(IPMA) and enzyme-linked immune sorbent assay (ELISA).Among these mAbs, 11H1 mAb specifically reacted with PRV gE/gI complex, as proven by western blot, IFA and IPMA. Determination of relative affinity results showed that 1G9, 11H1 and10D3mAbshad high relative affinity. Therefore, 1G9, 11H1 and 10D3 monoclonal antibodies may work as a useful tool for the development of differential diagnostic methods for PRV.

Introduction

Pseudorabies (Aujeszky's disease) is caused by pseudorabies virus and is an economically significant disease of swine characterized by central nervous system disorders, respiratory disease, reproductive failure and death [1]. PRV is a double-stranded DNA virus that belongs to the Alphaherpesvirinae subfamily of the Herpesviridae. The United States and some other countries has been successfully eradicated PRV by using glycoprotein E (gE) negative marker vaccines and blocking enzyme linked immunosorbent assay(ELISA) which could differentiate between infected animals and vaccinated animals [2,3].

PRV gE gene is 1734bplong in unique long section (ULS) of virus genome. The gE is a typical type membrane protein and the N-terminal 430 amino acids constitute extracellular region which has binding site with gI [4]. GE contains five uppermost antigenic epitopes in 52-238 amino terminals and two of these are conformation-dependent epitopes [5]. It is known that gE and gI are non-essential for replication of PRV and they form a non-covalently linked complex which is important for correct presentation of immunodominant epitopes on gE [6-9]. In this study, gE/gI complex was expressed in baculovirus expression system and corresponding mAbs were obtained by hybridoma technique [10].

The specificity and reactivity of mAbs were evaluated by indirect immune fluorescence assay and immunoperoxidase monolayer assay.

Materials and Methods

1. Vector and cell line

The truncated PRV gE gene (extracellular region) and gI gene (extracellular region) were amplified separately by PCR and inserted into pFastBacI plasmid (Invitrogen, Carlsbad, CA). The plasmid was transformed into DH10Bac cells and the white recombinant bacmids were chosen and subjected to bacmid DNA isolation. The recombinant bacmid DNA of gE and gI were used to transfect synchronously into Sf9 insect cells for subsequent gE/gI complex expression [11]. After 72 h, the P1 generation of virus was harvested. The P4 generation of virus was used to infected High Five cells at cell density 2×106cell/mL with 0.1 Multiplicity of Infection (MOI) and the gE/gI complex was harvested in the culture supernatant after 72 h. The gE/gI complex was then purified by immunoaffinity chromatography using Ni-NTA His Bind Resin (Novagen, Madison, WI) in accordance with the manufacturer's instructions. High Five cells were grown in Express Five SFM media supplemented with antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).

2. Immunization of mice

Four six-week-old female BALB/c mice were immunized with purified gE/gI complex (80 μg/mouse) emulsified in equal volume of Freund's complete adjuvant through hypodermic injection on the back. Two booster immunizations with the same dose of gE/gI complex plus Freund's incomplete adjuvant were administered at 2-week intervals. A final immunization with 100μg antigen without adjuvant was given by intraperitoneal injection 3 days before euthanasia [12].

3. Generation and purification of anti- gE/gI complex - specific mAbs

After four immunizations, anti-gE/gI complex serum titer of mice was determined by indirect ELISA [13]. Spleen from the immunized mice with the highest anti-gE/gI serum antibody titer were harvested and splenocytes were prepared in RPMI-1640 medium. The splenocytes were fused with SP2/0 myeloma cells at a ratio of 10:1 in RPMI-1640 medium by using 50% PEG 1500.The hybridoma cells were incubated in 96-well plates at 37°Cwith 5% CO2 in HAT screening culture medium. The supernatant of the fusion cells was subjected to indirect ELISA to check gE/gI antibody production. Positive hybridomas were cloned for at least three times by limiting dilution. The hybridoma cells were injected into paraffin-treated BALB/c mice to achieve ascetic fluid [12]. A total of 22 monoclonal antibodies were identified, and their immunoglobulin subclasses were determined by mouse mAb isotyping kit (Sigma, St. Louis, MO). Purification of mAbs was performed according to affinity binding with protein G resin method as described previously [14].

4. Western blot analysis

The reactivity of anti-gE/gI mAbs were analyzed using a western blot assay. The purified gE/gI complex was separated on 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (NC) membrane. The membrane was blocked with 5% skim milk for 12 h in PBST buffer(phosphate buffered saline [PBS] containing 0.05% Tween-20), then incubated with corresponding mAbs(1:100 diluted in PBST) for 2 hours at room temperature. The membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:5000 diluted in PBST) at room temperature for 1.5 h after washing three times with PBST. After washing five times, the substrate 3, 3-Diaminobenzidine Tetrahydrochloride Hydrate (DAB) was used to develop the signal [14].

5. Antibody titers of ELISA

Supernatants of hybridoma cell lines and mAbs were determined by indirect ELISA as previously described [13]. For supernatant of hybridoma cell lines, gradient dilutions of supernatant were added into cell wells starting from 1:800. For mAbs, gradient dilutions of mAbs were added to the cell wells starting from 1:10,000.

6. Indirect immunofluorescence assay and antibody titers of IFA

PK-15 cells were infected with PRV HN1201 strain at a MOI0.1 and cultured in DMEM at 37 °C for 48 h until cytopathic effect (CPE) appeared. Cells were washed once with PBS and were fixed in 80% cold acetone for 30 min at 4°C. Then cells were allowed to react with mAbs or hybridoma supernatant for 1 h. After washing three times with PBS, cells were then incubated with FITC-conjugated goat anti-mouse IgG (1:400 dilutions in PBS) for another 1 h followed by three washes with PBS. Finally, cells were observed under fluorescence microscopy. PK-15 cells infected with gE-deleted PRV were set as negative control.

MAbs and supernatants of hybridoma cell lines were serially diluted for detection of antibody titers of IFA. For supernatant of hybridoma cell lines, gradient dilutions of supernatant were added to the cell wells starting from 1:10. Form Abs, gradient dilutions of mAbs were added to the cell wells starting from 1:800.

7. Immunoperoxidase monolayer assay and antibody titers of IPMA

The confluent monolayer of PK-15 cells infected with PRV HN1201 (0.1MOI) or gE-deleted PRV were added into the wells and fixed in 80% cold acetone for 10 minutes at 4°C. After air drying, the plates were stored at -20°C until use. Supernatants of hybridoma cell lines andsp2/0 cells were diluted in PBS (0.01 mol/L, pH 7.2) and 5 μl were added to respective wells and incubated at 37°C for 50 minutes in wet box. After washing three times with PBS, 1:400 dilution of HRP-conjugated goat anti-mouse (Sigma) was added and incubated at 37°C for 40 minutes. The substrate 3-amino-9-ethylcarbazole was added after washing three times and incubated for 7 minutes at room temperature. After washing three times, 1:50 dilution of haematoxylin was added. After 10 seconds, the plates were washed with water. The plates were then examined under an inverted light microscope [15].

Supernatants of hybridoma cell lines were serially diluted for detection of antibody titers of IPMA. For supernatant of hybridoma cell lines, gradient dilutions of supernatant were added to the cell wells starting from 1:10.

8. Determination of relative affinity of mAbs

The relative affinity of mAbs was determined by competitive ELISA. In brief, polystyrene microtiter plates were coated with gE/gI complex (20 ng/well) at 4°C overnight. The plates were blocked with 150 μl 5% skim milk (BD Difco) in PBST for 2h at 37°C. A100 μl of 1:5000 dilution mAbs or SP2/0 cells ascites were added per well and allowed to react with doublegradient dilution of gE/gI complex in PBS (13800,6900,3450,1725,862.5,431.3,215.6,107.8,53. 9,27,13.5,6.7ng/ml,and 0 ng/ml) at 4°C for 24 h in 1:1 proportion. One hundred microliters of this mix was then added into the wells of microtiter plates and incubated at 37°C for 1h. After washing three times with PBST, 100 μl of 1: 10,000dilution HRP-conjugated goat anti-mouse (Sigma) was added to the wells and incubated at 37°C for 30 minutes. After washing with PBST three times, 100 μl of the substrate solution (0.2 mg/mL of TMB and 0.2% H2O2 in 0.05 mol/L citrate buffer, pH 4.6) was added, and the colorimetric reaction was developed for 15 minutes away from light at room temperature. The reaction was stopped by 50 μl of 2 mol/L sulfuric acid, and the value of optical density (OD) was measured at 450 nm. The relative affinity was calculated by A0/Ai=1+Ka×Ii, A0 was OD of the well of 0 ng/ml, Ai was OD of the well of 13800 ng/ml, Ii was 9.2×10-8mol/L.

9. Experiment of monoclonal antibody blocking positive serum

In short, the polystyrene microtiter plates were coated with the gE/gI complex (20 ng/well) at 4°C overnight. The plates were then blocked with 150 μl of 5% skim milk (BD Difco) in PBST for 2h at 37°C. One hundred microliters of 1:1000 dilution mAbs ascites and PBS were respectively added to the wells of microtiter plates, and incubated at 37°C for 30 minutes. A1:100 dilution of PRV gE positive serum from pig immunized with E. coli-origin recombinant gE was added and incubated at 37°C for 30 minutes. After washing three times with PBST, 100 μl of 1:10,000 dilution HRP-conjugated rabbit anti-pig (Sigma) was added per well

and incubated at 37°C for 30 minutes. After washing with PBST three times, 100 µl of the substrate solution (0.2 mg/mL of TMB and 0.2% H2O2 in 0.05 mol/L citrate buffer, pH 4.6) was added, and the colorimetric reaction was developed for 15 minutes away from light at room temperature. The reaction was stopped by 50 µl of 2 mol/L sulphuric acid, and the value of optical density (OD) was measured at 450 nm.

Results

1. Expression and purification of gE/gI complex

The expression plasmid containing gE or gI gene fragment in the correct direction and reading code frame were confirmed by the sequencing results (data no shown).The gE or gI plasmid were respectively transformed into DH10Bac E. coli cells and the positive recombinant bacmid were chosen by blue-white spot screening. Then, the P4 generation virus was harvested and inoculated into High Five cells. The recombinant gE/gI complex was expressed in the culture supernatant. Finally, it was purified by His-immunoaffinity chromatography and identified by SDS-PAGE analysis (Figure 1).

2. Preparation of monoclonal antibody

After three booster immunizations, mice sera antibodies against gE/gI complex were detected by indirect ELISA. All four immunized mices had high antibody titers ranging from 1:4×104 to 1:1.6×105.Mouseno. 4 with the highest antibody titer was chosen to fuse for further studies.

Three days after final immunization, the spleen cells were fused with SP2/0 myeloma cells by polyethylene glycol. Supernatant from single clones were detected by indirect ELISA and positive wells were subcloned for 4 cycles by limiting dilution. Finally, as shown in Table1, 22hybridoma cell lines producing monoclonal antibodies recognizing gE/gI complex were selected. Ascites were purified by protein affinity chromatography, and the concentrations of mAbs were shown in (Table1).

3. Antibody titers of ELISA

Antibody titers of ELISA of 22 mAbs were measured. Eight mAbs with high titers of monoclonal antibody cells supernatant and ascites were shown in (Table 2). Antibody titer of ELISA was the highest dilution of mAb which OD450greater than0.2. The highest titer of monoclonal antibody (5B10) supernatant and ascites were 1:12800 and 1:5.12×106.

Table 1: Subclass and concentrations of purified monoclonal antibodies.

hybridoma cell lines	subclass of mAb		Concentrations (mg/mL)
	heavy chain	light chain	
10D3	IgG2a	κ	2.9
11H1	IgG1	κ	2.8
12H4	IgG2b	κ	2.4
16F12	IgG1	κ	2.7
16G2	IgG1	κ	2.5
1B2	IgG2a	κ	2.2
1D12	IgG1	κ	2.8
1G9	IgG1	κ	3.0
20C5	IgG2a	κ	2.2
23H4	IgG2b	κ	2.7
2C12	IgG1	κ	2.3
2C6	IgG1	κ	2.5
2E12	IgG1	κ	2.7
2G10	IgG2b	κ	2.9
2G3	IgG1	κ	2.2
3D7	IgG2a	κ	2.4
3F12	IgG1	κ	2.5
4A3	IgG1	κ	2.9
5B10	IgG1	κ	2.1
5C9	IgG2b	κ	2.4
5E10	IgG1	κ	2.7
5F9	IgG1	κ	2.5

4. Antibody titers of IFA and IPMA

The antibody titers of IFA and IPMA were the highest dilution of positive result of mAbs. The antibody titers of IFA and IPMA of 22 mAbs were measured. Eleven mAbs with high antibody titers of IFA of monoclonal antibody cell supernatants and ascites were shown in (Table 3). The highest titers of monoclonal antibody cell supernatant and ascites were 1:80 and 1:12800. Similarly, 12 mAbs with high antibody titers of IPMA of monoclonal antibody cell supernatants were shown in (Table 4).The highest titer of monoclonal antibody cell supernatant was 1:160.

5. Specificity and reactivity of mAb11H1

Since mAb11H1 has the highest antibody titer of IFA and IPMA, specificity of mAb11H1 was analyzed by western blot, IFA and IPMA.As shown in (Figure 2), 11H1 mAb recognized recombinant gE/gI complex with two

bands at estimated protein molecular weight. In comparison, the supernatant of SP2/0 myeloma cells didn't react with gE/gI complex. To further test the specificity of mAb11H1, IFA and IPMA was used to test the cross-reactivity between 11H1 and PRV infected cells or gE-deleted PRV infected cells. As shown in (Figure 3), strong green fluorescence was observed in PK-15 cells infected by PRV. By contrast, no obvious fluorescence was observed in gE-deleted PRV infected cells. Also, the supernatant from SP2/0 myeloma cells were negative in IFA analysis. As shown in (Figure 4), PK-15 cells infected by PRV showed a strong red staining. There was no visible staining in gE-deleted PRV infected cells. Therefore, the above results confirmed the desirable specificity and reactivity of 11H1 mAb to PRV gE/gI complex.

Table 2: Antibody titer of ELISA of supernatants of hybridoma cell lines and ascites.

hybridoma cell lines	antibody titers of ELISA of cells supernatants	antibody titers of ELISA of ascites
5B10	1 : 12800	1 : 5.12×10^6
2G3	1 : 6400	1 : 2.56×10^6
2G10	1 : 6400	1 : 2.56×10^6
3F12	1 : 6400	1 : 2.56×10^6
4A3	1 : 6400	1 : 2.56×10^6
5C9	1 : 6400	1 : 2.56×10^6
5E10	1 : 6400	1 : 2.56×10^6
10D3	1 : 6400	1 : 2.56×10^6

Table 3: Antibody titer of IFA of supernatants of hybridoma cell lines and ascites.

hybridoma cell lines	antibody titers of IFA of cells supernatants	antibody titers of IFA of ascites
11H1	1 : 80	1 : 12800
5E10	1 : 80	1 : 6400
10D3	1 : 80	1 : 6400
1B2	1 : 40	1 : 3200
1D12	1 : 40	1 : 3200
1G9	1 : 40	1 : 3200
2C6	1 : 40	1 : 3200
2E12	1 : 40	1 : 3200
2G3	1 : 40	1 : 3200
3D7	1 : 40	1 : 3200
5F9	1 : 40	1 : 3200

Table 4: Antibody titer of IPMA of supernatants of hybridoma cell lines.

hybridoma cell lines	antibody titers ofIPMA of cells supernatants
10D3	1 : 160
11H1	1 : 160
1G9	1 : 160
2C6	1 : 160
2E12	1 : 160
5E10	1 : 160
12H4	1 : 80
16F12	1 : 80
16G2	1 : 80
20C5	1 : 80
2C12	1 : 80
4A3	1 : 80

Table 5: Results of relative affinityofmonoclonal antibodies.

hybridoma cell lines	OD_{450}		relative affinity Ka(mol/L)
	0 ng/mL	13800 ng/mL	
10D3	3.126	0.130	2.5×10^{-8}
11H1	3.013	0.116	2.7×10^{-8}
1G9	2.996	0.113	2.8×10^{-8}

Table 6: Results of experiment of monoclonal antibody blocking positive serum.OD2 equaled.

hybridoma cell lines	OD_{450}		OD1	blocking rate
10D3	0.782		0.751	49.00%
11H1	0.548		0.985	64.30%
1G9	0.595		0.938	61.20%
positive serum	1.547	1.519	/	/

6. Determination of relative affinity of mAbs

MAbs were used to determine the relative affinity of antibodies. The OD450 value of22 mAbs reacted with gE/gI complex of double gradient dilution. As shown in (Table 5), the higherrelative affinities of 1G9、11H1 and 10D3 mAbswere2.8×10-8 、 2.7×10-8 and 2.5×10-8mol/L, respectively. By contrast, the SP2/0 ascites did not react with gE/gI complex.

7. Experiment of monoclonal antibody blocking positive serum

10D3, 11H1 and 1G9mAbswith high relative affinity were chosen to make experiment of monoclonal antibody blocking positive serum. As shown in (Table 6), the block rates of 10D3, 11H1 and 1G9 mAbs ascites to positive serum were 49.0%、64.3% and 61.2%, respectively.

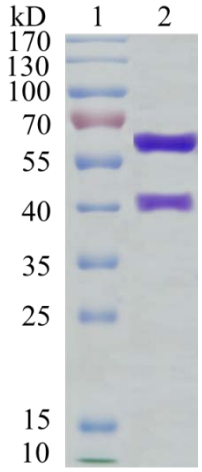

Figure1: Expression and purification of gE/gI complex and analysis by SDS-PAGE. Lane 1, protein marker; lane 2, purified gE/gI complex using immunoaffinity chromatography.

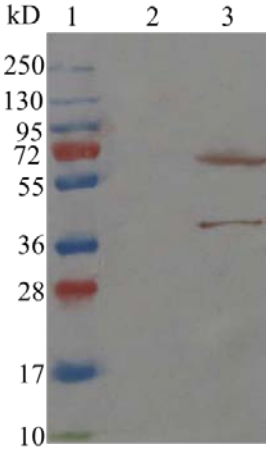

Figure 2: Specificity of monoclonal antibody 11H1 against gE/gI complex analyzed by western blot. Lane 1, protein marker; lane 2, supernatant of SP2/0 cells does not react with gE/gI complex; lane 3, 11H1 mAbreacts with gE/gI complex.

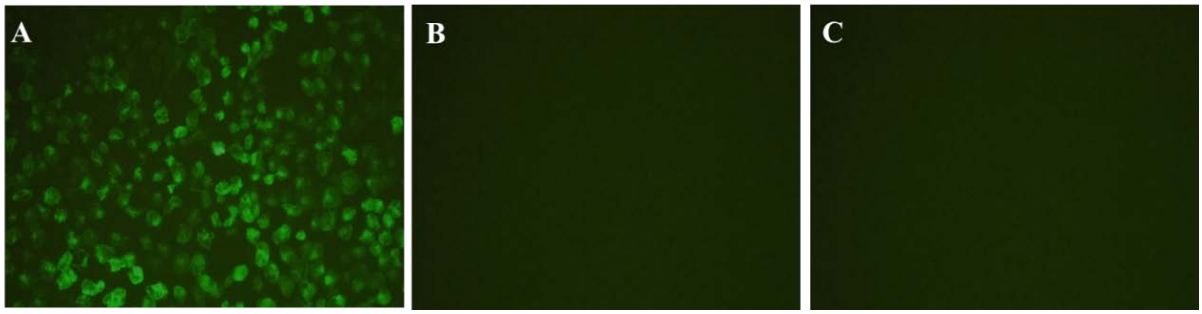

Figure 3: Reactivity of monoclonal antibody against gE/gI complex analyzed by IFA. (A) 11H1 mAbreacts with PK-15 cells infected by PRV; (B) 11H1 mAbdoes not react with PK-15 cells infected by gE-deleted PRV; (C)supernatant of SP2/0 cells does not react with PK-15 cells infected by PRV.

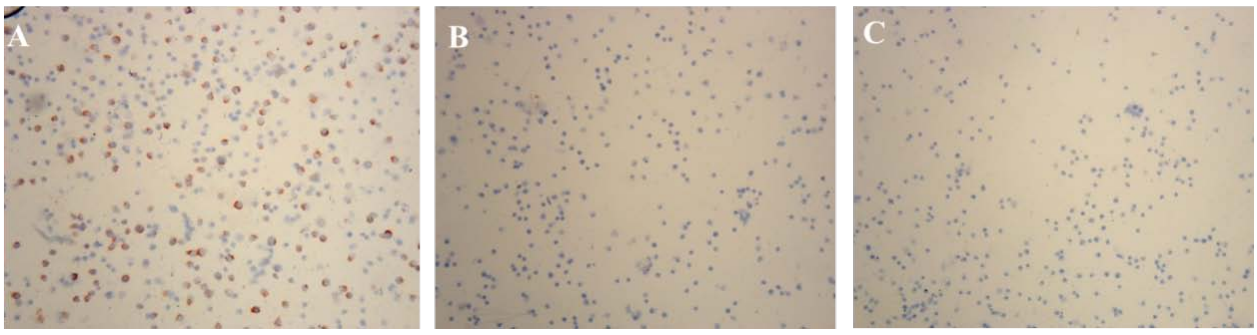

Figure 4: Reactivity of 11H1 monoclonal antibody against gE/gI complex analyzed by IPMA. (A) 11H1 mAbreact with PK-15 cells infected by PRV; (B) 11H1 mAbdoes not react with PK-15 cells infected by gE-deleted PRV; (C)supernatant of SP2/0 cells does not react with PK-15 cells infected by PRV.

Discussion

Since the emergence of PRV in the middle of 1800s, the outbreaks of PRV have been reported worldwide that led to huge economic loss to the swine industry. The gE encoded by US8 gene is an important target for vaccine and diagnostic reagent development. Commercial vaccines which contain gE-deleted PRV have been widely used [16,17]. Monitoring of gE-specific antibodies works as an important parameter for the disease diagnosis. Therefore, the raw reagents such as mAbs specific to PRV gE are necessary to establish the diagnostic methods.

Recombinant gE expressed in E. Coli was previously used as immunogen to prepare gE-specific mAbs. However, due to the lack of native conformation and glycosylation modification of the antigen expressed in prokaryotic system, the specificity and reactivity of mAbs to PRV were too low to be used for in-depth study. In PRV virions, same as in PRV-infected cells, gE and gI form non-covalent dimmers and work as functional and antigenic entity [6-9]. Therefore, gE/gI complex expressed by baculorvirus expression system was a preferred immunogen and was advantageous to elicit high levels of gE/gI-specific antibodies in immunized mice due to the natural conformation and glycosylation modification of recombinant gE/gI complex.

IFA and IPMA results showed 11H1 mAb has good reactivity with PRV at a cellular level. This confirmed that 11H1 mAb could recognize natural gE/gI complex and have no cross reactivity with other structural proteins of PRV. Experiment of monoclonal antibody blocking positive serum indicated the recognized epitope of the 10 D3, 11H1 and 1G9mAbs were similar to dominating antibody in serum. This deduced the epitopes which 10D3, 11H1 and 1G9 recognized PRV could be B cell dominant epitopes of PRV.

In conclusion, in this study, we established a panel of monoclonal antibody which included 22 mAbs against gE/gI complex.10D3, 4A3 and 5E10 mAbs could be used for IFA, 1G9, 11H1, 10D3, 2C6, 2E12 and 5E10 mAbs could be used for IPMA, and 5B10, 2G3 mAbs could be used for ELISA.10D3, 11H1 and 1G9mAbswere sufficiently to enable the use of an blocking ELISA for the detection of antibodies directed against gE in swine sera and further functional analyses of gE. Therefore, the above mAbs may work as valuable tools for biological studies of PRV.

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