Short communication

Generation of monoclonal antibodies and epitope mapping of ApxIV A of *Actinobacillus pleuropneumoniae*

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Abstract

To study functions of ApxIV, a species-specific and in vivo inducible RTX toxin identified in *Actinobacillus pleuropneumoniae* recently, and to develop a diagnostic trial distinguishing the pigs infected naturally and vaccinated with inactivated and/or subunit vaccines, we attempted to prepare monoclonal antibodies against ApxIV. BALB/c mice were immunized with ApxIV AN and ApxIV AC which are N- and C-terminal halves (814 and 997 amino acids, respectively) of ApxIV A produced in *E. coli* BL21 (DE3), respectively. Eight monoclonal antibodies were selected, four (designated as 1A8, 1G5, 3E7 and 4H9) against ApxIV AN and another four (named as 1B12, 2E5, 4D8 and 4G2) against ApxIV AC. Western blot and ELISA additivity assays suggested that all monoclonal antibodies except 1A8 are specific to the corresponding immunogen, 1A8 reacts with both immunogens which have an overlapping region of 156 residues. ELISA additivity tests revealed that at least five epitopes in ApxIV are defined by eight monoclonal antibodies, two between 1 and 866 amino acids, one between 867 and 1022 amino acids and two between 1023 and 1863 amino acids. In conclusion, we have succeeded in producing eight monoclonal antibodies, which react with five different epitopes of ApxIV.

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Keywords: *Actinobacillus pleuropneumoniae*; ApxIV; Monoclonal antibody; ELISA additivity

1. Introduction

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine contagious pleuropneumonia which occurs worldwide and causes great economic losses in the pig industry. To date, 15 serotypes of APP (Blackall et al., 2002) have been described which variously express four different RTX toxins, ApxI, ApxII, ApxIII and ApxIV (Frey, 1995; Schaller et al., 1999). Many lines of evidence suggested that ApxI, ApxII and ApxIII play a predominant role in pathogenesis (Frey, 1995; Kamp et al., 1997; Boekema et al., 2004). ApxI is a 110kDa protein with strongly haemolytic and cytotoxic activities, secreted by the most virulent serotypes 1, 5, 9, 10, 11 and 14. ApxII is a protein of 105kDa expressed by all serotypes except for serotypes 10 and 14, and is moderately haemolytic and cytotoxic. ApxIII is a 120kDa protein, produced by serotypes 2, 3, 4, 6, 8 and 15, and has strongly cytotoxic but non-haemolytic activity (Kamp et al., 1991; Frey, 1995).

ApxIV is the fourth RTX toxin identified in APP recently, its biologic activity and function is not clear except that recombinant ApxIV produced in *E. coli* BI21 (DE3), respectively. Eight monoclonal antibodies were selected, four (designated as 1A8, 1G5, 3E7 and 4H9) against ApxIV AN and another four (named as 1B12, 2E5, 4D8 and 4G2) against ApxIV AC. Western blot and ELISA additivity assays suggested that all monoclonal antibodies except 1A8 are specific to the corresponding immunogen. 1A8 reacts with both immunogens which have an overlapping region of 156 residues. ELISA additivity tests revealed that at least five epitopes in ApxIV are defined by eight monoclonal antibodies, two between 1 and 866 amino acids, one between 867 and 1022 amino acids and two between 1023 and 1863 amino acids. In conclusion, we have succeeded in producing eight monoclonal antibodies, which react with five different epitopes of ApxIV.

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ApxIV using the Mabs. These will contribute to study the structure, function and immunological characteristics of ApxIV and to develop serologic differential diagnostic methods.

2. Materials and methods

2.1. Bacteria strains and culture conditions

E. coli strains DH5α and BL21 (DE3) were used for cloning and expression of cloned genes. APP reference strains 1421 (serotype 3), WFS8 (serotype 7), 1421 (serotype 10), WF83 (serotype 7) and 1421 (serotype 3) respectively with 50% saturated aminosulfate. The bacteria were kindly donated by Dr. P. Blackall and Dr. R. Bowles (Australia). The field strain of APP (0306SYML, serotype 1) was isolated by our laboratory. All bacteria were cultured at 37 °C on Tryptic soy agar (TSA, DIFCO, USA) with 0.01% NAD or in Tryptic soy broth (TSB; DIFCO) supplemented with 0.01% NAD and 1 mM CaCl₂.

2.2. Production of recombinant ApxIV

In order to construct plasmids encoding poly-histidine-tailed ApxIV fusion proteins, we amplified two segments from genomic DNA of strain 0306SYML, respectively, using the following primers designed according to the apxIV sequence of serotype 1 (GenBank accession no. AF021919): apxIVA (5′-cgcCATATGgagcgactttctctggcgc-3′), apxIVA (5′-cgcCATATGggcgat-ttaaatttcag-3′). The PCR products were digested with NdeI and HindIII for cloning into pET-28b. The resulting plasmids, designated as pETapIVA5#1 (5′-cgcCATATGggcgat-ttaaatttcag-3′), pETapIVA5#2 (5′-cgcCATATGgttactctctctctct-3′). The PCR products were purified from agarose gels and subsequently digested with NdeI and HindIII for cloning into pET-28b. The resulting plasmids, designated as pETapIVA5, were transformed into BL21 (DE3) successfully, and ApxIV AN and ApxIV AC were expressed in E. coli BL21 (DE3) for expression of the fusion proteins. The pETapIVA5 expressed the 90 kDa N-terminal half of ApxIV, designated as ApxIV AN, and the pETapIVA5 expressed the 115 kDa C-terminal half of ApxIV, designated as ApxIV AC. The inclusion bodies were extracted as described by Marshak et al. (1996). The expression of ApxIV AN and ApxIV AC was analyzed by Western blot.

2.3. Preparation of monoclonal antibodies against ApxIV

Female BALB/c mice, 4–6 weeks of age, were immunized subcutaneously with 50 μg ApxIVAN and ApxIVAC emulsified with Freund’s complete adjuvant (Sigma, USA) in 0.2 ml, respectively, on day 0, and boosted twice on day 14 and 28 with the same dose of antigens emulsified with Freund’s incomplete adjuvant (Sigma). The antibody levels were examined by an enzyme-linked immunosorbant assay (ELISA) using the ApxIV AN and ApxIV AC as coating antigens. The mice with the highest serum antibody titer were injected intravascularly using 150 μg of antigen without adjuvant. Three days later, the splenocytes of the immunized mice were isolated and fused with SP2/0 Ag14 myeloma cells using 50% (v/v) polyethylene glycol (Sigma). Hybridoma were screened for secretion of desired antibodies by ELISA and cloned twice by limiting dilution method. Monoclonal antibody isotypes were determined by ELISA using horseradish peroxidase-conjugated IgG1 and IgG2a (Southern Biotechnology Association, USA). Large quantities of the monoclonal antibodies were produced by intraperitoneal injection of hybridoma cells into liquid paraffin-treated BALB/c mice. After 7–14 days, the ascites containing high concentrations of antibodies were harvested.

2.4. Determination of the monoclonal antibodies specificity

To determine the specificity of the Mabs, they were first analyzed by Western blot with recombinant proteins ApxIV AN and ApxIV AC, and native ApxI, ApxII and ApxIII which were purified from the overnight culture media of APP strains 22009 (serotype 10), WFS8 (serotype 7) and 1421 (serotype 3) respectively with 50% saturated aminosulfate. The cell lysates and cultural supernatants of several pig pathogens such as E. coli, Pasteurella multocida, Haemophilus parasuis and Bordetella bronchiseptica were also used to test the cross-reactivity of the Mabs in Western blot. Protein samples were boiled for 5 min after mixing with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer, separated on 5% stacking/12% separating SDS-polyacrylamide gels, and electroblotted to nitrocellulose membrane (Sijia Biochemistry Plastic Co., China) using Trans-Biorad SD (Bio-Rad Laboratories). Each blot was washed once with TBS-T buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 10 min and blocked with 5% skimmed milk in TBS-T overnight. Then the blot was incubated with Mabs at room temperature for 30 min. After three times washing in TBS, the blot was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Association). 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate for membrane development.

Fig. 1. SDS-PAGE analysis of the expression of ApxIV AN and ApxIV AC. After induction by IPTG, two fusion proteins, ApxIV AN (lane 1) and ApxIV AC (lane 2), were expressed in E. coli BL21 (DE3) successfully, and ApxIV AN and ApxIV AC existed predominantly with inclusion bodies as shown by lane 3 and lane 4, respectively. Lysates of the BL21 (DE3) transformed by pET-28b (lane 2, lane 2) were as control. The sizes of molecular mass markers (lane M) were 116.0, 66.2, 45.0, 35.0 and 25.0 kDa, respectively, from the above to the bottom.
Table 1
Biological properties of eight anti-ApxIV A monoclonal antibodies

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Reference of MAb</th>
<th>Antibody isotype</th>
<th>Reactivity in ELISA with</th>
<th>Reactivity in WB with</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApxIV AN</td>
<td>1A8</td>
<td>G1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1G5</td>
<td>G2a</td>
<td>++++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>3E7</td>
<td>G2a</td>
<td>++++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>4H9</td>
<td>G2a</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>ApxIV AC</td>
<td>1B12</td>
<td>G1</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2E5</td>
<td>G2a</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>4D8</td>
<td>G2a</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4G2</td>
<td>G2a</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

2.5. ELISA additivity test

In order to test the Mabs recognizing different epitopes, ELISA additivity tests were performed as described by Friguet et al. (1983). The ApxIV AN and ApxIV AC were coated onto 96-well ELISA plates (Kangjia Ltd., China), respectively. Two Mabs were added either separately or simultaneously, and the amount of bound antibody was quantitatively measured. An additivity index (AI), which compares the ODs obtained by two assays (alone and in a mix) under standardized conditions, was calculated for each pair of Mabs according to the formula:

\[ AI = \frac{100 \times \left( \frac{A1 + A2}{2} \right) - 1}{100} \]

Here, \( A1 \) and \( A2 \) are the ODs obtained when the Mabs are assayed separately, and \( A1 + A2 \) is the OD when the same amounts of the two Mabs are pooled in the same well. Provided the concentrations of the Mabs are saturating for the antigen, the AI will tend to be zero if both Mabs recognize the same epitope, but close to 100 if the two epitopes are topographically unrelated.

3. Results and discussion

Two recombinant proteins, ApxIV AN and ApxIV AC, were expressed in E. coli BL21 (DE3) after induction by IPTG, which existed predominantly with inclusion bodies (see Fig. 1). The fusion proteins were purified from the inclusion bodies, denatured with sodiumlauryl sulfate and renatured by glutathione G-S-S-G. The immunogenicity of the purified ApxIV AN and ApxIV AC was analyzed by Western blot and ELISA with
Table 2
Additivity index for eight anti-ApxIV monoclonal antibodies

<table>
<thead>
<tr>
<th>Mab</th>
<th>IA8</th>
<th>IG5</th>
<th>IE6</th>
<th>4H9</th>
<th>1B12</th>
<th>2E5</th>
<th>4D8</th>
<th>4G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA8</td>
<td>85.5 (5.3)</td>
<td>88.5 (5.3)</td>
<td>59.6 (1.2)</td>
<td>59.1 (2.0)</td>
<td>-44.1 (-6.7)</td>
<td>-50.3 (-7.9)</td>
<td>-56.4 (-8.3)</td>
<td>-62.9 (-8.9)</td>
</tr>
<tr>
<td>IG5</td>
<td>12.1 (5.7)</td>
<td>76.3 (4.6)</td>
<td>27.2 (2.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IE6</td>
<td>-36 (1.0)</td>
<td>74.1 (4.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4H9</td>
<td>15.4 (3.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B12</td>
<td>11.1 (-2.1)</td>
<td>87.4 (-8.8)</td>
<td>54.6 (-4.9)</td>
<td>-10.7 (-7.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2E5</td>
<td>1.2 (-3.0)</td>
<td>-12.1 (-2.1)</td>
<td>-72.7 (-4.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4D8</td>
<td>-23.0 (-4.6)</td>
<td>-58.4 (-4.1)</td>
<td>-72.7 (-4.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4G2</td>
<td>-16.5 (-6.8)</td>
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</tbody>
</table>

ELISA additivity tests. Mean ELISA additivity indexes as calculated for paired Mabs in duplicate assays carried out according to Friguet et al. (1983). Numbers in parenthesis are standard deviation. Results placed in a same square indicate overlapping epitopes. All Mabs and paired Mabs were respectively assayed with antigen ApxIV AN and ApxIV AC. Their AIs were shown in a form of n/c, here n is the AI assayed by antigen ApxIV AN, and c is that by ApxIV AC. When there was no reaction the AI is indicated as "-". Only Mab IA8 reacted with both antigens.

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References


