Passive Protection of Mice against *Actinobacillus pleuropneumoniae* Infection by Monoclonal Antibodies

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**Abstract.** Mouse monoclonal antibodies (MAbs), Y-4b, F-6b and K-6a recognized as capsular antigens of *A. pleuropneumoniae* serotypes 1, 2 and 5, respectively, and MAb H-1b as lipopolysaccharide (LPS) of serotype 2 were produced. We examined the passive immunizing efficacy of these MAbs on *A. pleuropneumoniae* infection in C3H/HeJ mice. On the challenge infection of homologous serotype strains, they showed a sufficient protective effect in immunized mice. It was concluded that MAbs recognized as capsular antigen and LPS have a serotype-specific protective effect on *A. pleuropneumoniae* infection, suggesting the important role in preventing *A. pleuropneumoniae* infection. —**Key words:** *Actinobacillus pleuropneumoniae*, monoclonal antibody, passive immunity.

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**Actinobacillus pleuropneumoniae** (*A. pleuroneumoniae*) is one of the most important swine pneumonic pathogens, and the economic loss caused by acute infection and chronic pleuropneumonia by this organism is a major problem in the swine industry.

Recently, current vaccines containing inactivated bacterial cells of *A. pleuropneumoniae* are used to control and to prevent the disease. However, in the experimental infection, inactivated cells vaccine was confirmed to be incapable of preventing the subclinical or pathological manifestations and to have only serotype-specific protection [17, 21].

Many investigators have previously reported the protective effect of cell extract of *A. pleuropneumoniae*; capsular antigen, outer membrane proteins and lipopolysaccharide have a protective activity for *A. pleuropneumoniae* infection [4, 7–9, 18]. These cell extracts include some many kinds of bacterial components that there is a difficulty in identifying the specific protective antigen from them. In the present study, we produced mouse monoclonal antibodies (MAbs) to capsular antigen and LPS of *A. pleuropneumoniae*, and examined the protective effect by passive immunization of MAbs in C3H/HeJ mice.

**Materials and Methods**

**Bacterial strains:** *A. pleuropneumoniae* strains Y-1 (serotype 1), G-4 (serotype 2) and E-3 (serotype 5) were used. They were isolated from lung lesion of swine having symptoms of pneumonia in the field, and serotyped in our laboratory.

**Culture conditions:** The bacteria were cultured in modified Sawata’s-broth (S-broth) [13] containing 20% chicken meat infusion, 0.5% casamino acid (Difco Laboratories, Detroit, Mich., U.S.A.), 0.5% polypeptide (Wako Pure Chem. Ind., Osaka, Japan), 0.5% yeast extract (Difco) and 0.004% nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 6 hr at 37°C with shaking.

**Preparation of cell-free antigen (CFA):** The culture supernatant of three strains was collected by centrifugation at 11,000 × g for 20 min. Each supernatant was concentrated to 1/20 by negative pressure dialysis in phosphate-buffered saline (PBS), pH 7.4.

**Virulence of *A. pleuropneumoniae* strain Y-1, G-4 and E-3:** Various concentrations of strains Y-1, G-4 and E-3 in 0.2 ml of PBS were inoculated intraperitoneally to 8-week-old C3H/HeJ mice (Japan SLC Inc., Shizuoka, Japan). The 50% lethal dose (LD<sub>50</sub>) of each strain was calculated by the method of Reed and Muench [19].

**Purification of capsular antigen and LPS:** The capsular antigen was extracted from broth cultures of each strain by the method of Inzana [7, 8]. Briefly, bacterial cells cultured for 6 hr at 37°C with shaking were centrifuged at 11,000 × g for 20 min and the supernatant was recentrifuged. For the extraction of capsular antigen from the supernatant, N-cetyl-N,N,N-trimethylammonium bromide (Cetavlon Sigma) was added to make a final concentration of 0.01 M and the supernatant was incubated at 4°C for 24 hr. The precipitate was collected by centrifugation at 11,000 × g for 20 min and extracted with 0.4 M NaCl. Two volumes of 95% ethanol were added to the extract and the extract was incubated at −20°C for 24 hr. After the centrifugation at 11,000 × g for 20 min, the precipitate was resuspended with distilled water and dialyzed. The solution was then extracted by 90% phenol and the aqueous phase was dialyzed again in distilled water. Sodium chloride was added to the solution to make a final concentration 0.1 M and two volumes of 95% ethanol was then added. The precipitate formed was collected by centrifugation at 11,000 × g for 20 min and resuspended with distilled water as the capsular antigen.

The antigen was stored at 4°C until used.

We followed the procedure of Westphal and Jhan in isolating LPS [24]. In brief, bacterial cells were treated with acetic. Dried bacteria were extracted with phenol...
and aqueous phase was dialyzed in water. The fraction was digested with pronase (Sigma) at 37°C for 2 hr and centrifuged at 100,000 × g for 6 hr at 4°C. The pellet was resuspended with distilled water and centrifuged more two times for washing. LPS preparation in distilled water was lyophilized and stored at 4°C until used.

Preparation of mouse MAb: After the broth cultivation of each strain, 0.2 ml of formalized whole cell antigen adjusted to a concentration of 5.0 × 10⁸ colony-forming units (CFU)/ml was emulsified with an equal amount of Freund’s incomplete adjuvant and injected twice subcutaneously into 8-week-old BALB/c mice at intervals of two weeks. Three days before fusion, 0.2 ml (2.5 × 10⁸ CFU/ml) of formalized cell in PBS was injected intraperitoneally. The spleen cells from the immunized mice were fused at a ratio of 5:1 with mouse myeloma cells (SP2/0-Ag14) in the presence of 50% polyethylene glycol 4000 (Merck & Co., Inc., Darmstadt, FRG) following the method of Köhler and Milstein [10]. The hybridoma cells were selected in HAT medium containing hypoxanthine-aminopterin-thymidine at 37°C in 5% CO₂. Culture supernatant were screened by enzyme-linked immunosorbent assay (ELISA), and positive hybridoma cells were cloned by the limiting dilution. These cells were injected intraperitoneally to BALB/c mice, primed with pristane (Sigma) 3 weeks prior to the injection of cells. Ascites fluid collected from these mice was used as MAbs. The immunoglobulin subclass of MAbs was determined by Mouse monoclonal antibody isotyping kit (Amersham Japan, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting: Discontinuous SDS-PAGE was performed with a 3.8% stacking gel and a 10% separating gel and the two-buffer systems of Laemmli [14]. The cell free antigen (CFA) of each strain was solubilized by the treatment at 100°C for 3 min in sample buffer containing 2% SDS (Sigma), 5% 2-mercaptoethanol (Sigma), 10% glycerol (Wako) and 0.03% bromophenol blue (Wako) in 0.063 M Tris-hydrochloride buffer, pH 6.8. Electrophoresis was carried out at a constant voltage of 150 V until the dye front migrated to the end of the gel. After SDS-PAGE, the separated CFA were electrophoretically transferred to Immobilon membrane (Millipore, Bedford, MA, U.S.A.) by the following method of Towbin et al. [23]. The gels were soaked in transfer buffer (25 mM Tris-base, 192 mM glycine and 20% methanol) and then electrobotted onto Immobilon membrane for 4 hr at 36 V using a Transblot cell (Bio-Rad, Richmond, CA, U.S.A.). The membrane was blocked overnight with 5% gelatin (Bio-Rad) in Tris-buffered saline (TBS; 20 mM Tris, 0.5 M NaCl, pH 7.4) and then incubated with MAb for 1 hr at room temperature. The excess MAb was removed by washing three times in TBS containing 0.05% Tween 20 (T-TBS). The membranes were incubated for 1 hr with peroxidase-conjugated goat anti-mouse IgG, IgM and IgA (Cappel, West Chaster, PA, U.S.A.) diluted 1:2,000 with TBS. After washing three times with T-TBS, the membranes were incubated in color developing reagent (10 mg of 3-3’ diaminobenzidine and 10 μl of hydrogen peroxide in 50 ml of TBS).

ELISA: The CFA of each serotype was coated overnight in the well of microtiter plate with carbohydrate buffer (pH 9.6) at 4°C. The wells were washed with PBS containing 0.005% Tween 20 (T-PBS) and the MAbs of serotype by hybridoma or ascites fluid were added. The wells were then incubated for 1 hr at 37°C and washed three times with T-PBS. The wells to which the diluted peroxidase-conjugated goat anti-mouse IgG, IgM and IgA (1:2,000) were added were incubated for 1 hr at 37°C. After five times washing with T-PBS, the wells were developed with the substrate solution (20 mg of o-phenylenediamine and 20 μl of hydrogen peroxide in 50 ml of 0.05 M citric acid-0.1 M Na₂HPO₄ buffer pH 5.0) for 30 min at 25°C. The reaction was stopped by 1.0 M H₂SO₄, and the absorbance at a wave-length of 492 (A₄₉₂) was measured by the microplate reader (Sanko Pure Chem. Ind., Tokyo, Japan).

ELISA-inhibition test: Each MAb was preincubated for 30 min at 37°C with inhibitors consisting of purified capsular antigen and purified LPS from A. pleuropneumoniae serotype 1, 2 and 5. The mixture was added to each well of microtiter plates coated with the CFA of each serotype. ELISA was performed as described above. The ratio of inhibition was calculated as follows: 100 × (A₄₉₂ in absence of inhibitor — A₄₉₂ in presence of inhibitor) / A₄₉₂ in absence of inhibitor).

Passive immunizing efficacy of MAbs: Ascites fluid was diluted with PBS to be A₄₉₂ of approximately 1.0 in ELISA. C3H/HeJ mice were immunized intraperitoneally with 0.5 ml of each MAb. Twenty four hours later, immunized groups each consisting of 10 heads were challenged intraperitoneally with approximately 5-fold LD₅₀ of strain Y-1, G-4 or E-3 (2.0 × 10⁷, 5.0 × 10⁶ or 2.0 × 10⁷ CFU/head, respectively). The control groups were inoculated with 0.5 ml of ascites fluid containing porcine rotavirus MAb (IgM). The animals were observed for seven days after the challenge infection, and the number of surviving mice were recorded.

RESULTS

Characterization of MAb: The characteristics of supernatant or ascites fluid obtained from four different hybridomas are summarized in Table 1. All of these hybridomas formed IgM antibodies. The antigens recognized by MAbs were determined by the immunoblotting and ELISA-inhibition test. MAb Y-4b (serotype 1) reacted only to serotype 1 CFA, showing smear band in immunoblotting (Fig. 1-A). MAb F-6b (serotype 2) and MAb K-6a (serotype 5) also indicated serotype-specific reaction and smear bands (Fig. 1-B, D). MAb H-1b (serotype 2) reacted to serotype 2 CFA-specific and showed ladder bands characteristic of LPS (Fig. 1-C). The ratio of inhibition of binding between CFA and purified capsular antigen or purified LPS of each serotype is shown.
Table 1. Characterization of MAb

<table>
<thead>
<tr>
<th>MAb</th>
<th>Serotype</th>
<th>Immunoglobulin subclass</th>
<th>Recognized antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-4b</td>
<td>1</td>
<td>IgM</td>
<td>Capsular antigen</td>
</tr>
<tr>
<td>F-6b</td>
<td>2</td>
<td>IgM</td>
<td>Capsular antigen</td>
</tr>
<tr>
<td>H-1b</td>
<td>2</td>
<td>IgM</td>
<td>LPS</td>
</tr>
<tr>
<td>K-6a</td>
<td>5</td>
<td>IgM</td>
<td>Capsular antigen</td>
</tr>
</tbody>
</table>

a) The immunoglobulin subclass of MAb was determined by a mouse monoclonal antibody isotyping kit.
b) The recognized antigens of MAb to A. pleuropneumoniae were determined by the immunoblotting and ELISA-inhibition test.

Table 2. ELISA-inhibition test using capsular antigen or LPS purified from A. pleuropneumoniae serotype 1, 2 and 5

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Serotype</th>
<th>MAb</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsular</td>
<td></td>
<td>Y-4b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-6b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-1b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-6a</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td></td>
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<td>2</td>
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<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

The procedure of the ELISA-inhibition test is described in Materials and Methods.

in Table 2. Purified capsular antigen inhibited the binding of MAb Y-4b, F-6b and K-6a to each serotype CFA (inhibition rate: 80%-100%). MAb H-1b revealed the high inhibition rate (97%) by purified LPS from serotype 2 (Table 2). As a result, MAb Y-4b, F-6b and K-6a recognized each serotype capsular antigen and MAb H-1b recognized LPS of serotype 2.

Virulence of strains: The mortality in C3H/HeJ mice inoculated intraperitoneally with 3 strains of A. pleuropneumoniae was dose-responsive as shown in Table 3. The LD_{50} of strains Y-1, G-4 and E-3 were to 5.0 × 10^6, 1.0 × 10^9 and 5.0 × 10^6 CFU, respectively.

Protection after passive immunization: Mice immunized passively with MAb Y-4b to capsular antigen of serotype 1 showed a high survival rate (8/10) against the challenge of the homologous serotype, although no mouse could tolerate challenge of the heterologous serotype. In addition, protective efficacy of MAb F-6b and K-6a was observed only against the challenge of homologous serotype (survival rate 7/10, 9/10), not against heterogeneous serotype. MAb H-1b to LPS of serotype 2 showed perfect protection against only the same serotype in immunized mice. On the other hand, none of the control groups survived.

Table 3. Virulence of the strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Challenge dose (CFU/Head)</th>
<th>Survival rate</th>
<th>LD_{50} (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-1</td>
<td>1</td>
<td>1.0 × 10^6, 1.0 × 10^7, 1.0 × 10^8</td>
<td>0/10</td>
<td>5.0 × 10^6</td>
</tr>
<tr>
<td>G-4</td>
<td>2</td>
<td>1.0 × 10^9</td>
<td>0/10</td>
<td>1.0 × 10^6</td>
</tr>
<tr>
<td>E-3</td>
<td>5</td>
<td>1.0 × 10^6, 1.0 × 10^7, 1.0 × 10^9</td>
<td>0/10</td>
<td>5.0 × 10^6</td>
</tr>
</tbody>
</table>

a) Number of mice surviving/inoculated. The LD_{50} of each strain was determined according to the method of Reed and Muench.

Table 4. Passive immunizing efficacy of 4 MAb

| Strain | Serotype | Challenge dose (CFU/Head) | Survival rate | Y-4b | F-6b | H-1b | K-6a | Comb |
|--------|----------|---------------------------|---------------|------|------|------|------|--|------|
| Y-1    | 1        | 2.0 × 10^12                | 1/10          | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| G-4    | 2        | 5.0 × 10^12                | 2/10          | 0/10 | 1/10 | 1/10 | 0/10 | 1/10 |
| E-3    | 5        | 2.0 × 10^12                | 2/10          | 1/10 | 1/10 | 1/10 | 0/10 | 1/10 |

a) Number of mice surviving/inoculated.
b) MAb against procine rota virus.
C3H/HeJ mice passively immunized intraperitoneally with MAb and challenged with A. pleuropneumoniae 24 hr after immunization.
DISCUSSION

Many investigators have studied the protective antigens of *A. pleuropneumoniae* [4, 11, 12, 15, 22], suggesting that the capsular antigen, the LPS and the outer membrane proteins were important protective antigens in *A. pleuropneumoniae*. However, these substances have not been evaluated sufficiently since the purification of the bacterial component is difficult, and the purification is often accompanied by the loss of immunological activity. Recently, it has been reported that the passive immunization technique using MAbs is suitable for screening the protective effect of bacterial components [12], and that C3H/HeJ mice are useful for the immunological study of gram negative bacteria, since these mice did not respond to the endotoxin activity, mitogenicity or adjuvanticity of LPS [6]. We have postulated that capsular antigen and LPS from *A. pleuropneumoniae* may be protective antigens as a result of a passive immunity using MAbs in C3H/HeJ mice.

Capsular antigen and LPS are recognized as serotype-specific antigens in many other bacterial species. The identity of the serotype-specific antigen of *A. pleuropneumoniae* has been proposed to be either the capsule or the LPS, or both [1–3, 5, 16]. Our MAbs recognized as capsular antigen and the LPS showed serotype-specific reaction in the immunoblotting and ELISA-inhibition test. However, in ELISA-inhibition test, these MAbs cross-reacted only slightly. The reaction was caused by using antigen for inhibitor mingling with a few common antigens of *A. pleuropneumoniae*.

Nielsen [17] has reported that immunization with live *A. pleuropneumoniae* provided a protective effect on heterogeneous serotypes, and that immunization with a killed whole cell vaccine-induced serotype-specific immunity only. Nielsen also showed that intranasal vaccination by live antigen in pigs provided some protective effect on infection by the heterogeneous serotypes of the bacteria, and that no serum antibodies to heterogeneous serotypes were found. Inzana et al. [8, 9, 21] showed that the immunogenicity of purified *A. pleuropneumoniae* capsular material was very poor in rabbits and pigs compared with that of capsular polysaccharides on the bacteria. The immunogenicity of the capsule might possibly be improved by conjugation to a protein carrier. Capsule is generally considered to be an important factor associated with the virulence of bacteria [20]. Encapsulated bacteria are able to resist bactericidal action in serum and to avoid phagocytic systems in the absence of specific antibody. However, specific antibody to capsular antigen stimulates opsonification against an invaders in the host. The presence of type-specific antibody to capsular antigen is therefore sufficient to develop clinical symptoms in the host after infection including *Haemophilus influenzae* type B, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Escherichia coli*, and others [20].

The present study showed that antibodies to the capsular antigen and LPS of *A. pleuropneumoniae* have a protective effect on lethal challenge dose in mice. It was suggested that these antigens which induced these antibodies might be valuable constituents of component vaccine for bacterial pneumonia due to *A. pleuropneumoniae* infection.

REFERENCES

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