Production of Monoclonal Antibody against Pasteurella multocida Strains X-73 and P-1059

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Abstract Monoclonal antibodies (mAbs) of the predominant strains of fowl cholera were prepared. The mAbs were prepared by immunization of BALB/c mice with a formalinized whole-cells of Pasteurella multocida strains P-1059 (serovar A:3) or X-73 (serovar A:1). Totally three hybridomas producing mAb against strain P-1059 (YamaP1-3) and one hybridoma against strain X-73 (Mame1) were obtained. Immunoblot analysis of the hybridomas revealed that all the mAbs recognized only one epitope of each strain and showed no cross reaction among 16 somatic serotype of P. multocida. The N-terminal analyses indicated that mAb YamaP1 recognized the OmpH while mAb Mame1 recognized the OmpA. Treatment of crude capsular extraction (CCE) or detergent insoluble fraction outer membrane protein (DIF-OMP) antigen with proteinase K or periodic acid indicated that the epitope recognized was proteinaceous. Mice passively immunized with the mAbs showed no cross protectivity by heterologous challenge exposure.

Keywords: Pasteurella multocida, Monoclonal antibody, P-1059, X-73

Introduction Pasteurella multocida is a gram negative bacterium and the causative agent of fowl cholera, bovine or buffalo hemorrhagic septicemia and swine atrophic rhinitis. Bacteria can be classified into 5 capsular serogroups A, B, D, E and F (¹) and 16 somatic serotypes 1-16 (²). Capsular serogroup A and somatic serotypes 1, 3 and 4 are known as the causative agents of fowl cholera (¹). The virulence factors of P. multocida including capsular protein have been demonstrated (³). Bacterial capsule plays a role to adhere to epithelial cells of host cells at the early stage of infection (⁴). The previous studies demonstrated that monoclonal antibody (mAb) against 39 kDa
protein in crude capsular extract (CCE) from strain P-1059 (serovar A:3) exhibited cross-reactivity to the extract from serovar A:1 (strain X-73) \(^5\). The mAb to major somatic serotype for fowl cholera is needed to be developed. Then, the aims of this study were to produce the serotype specific mAb for \textit{P. multocida} serotype 1 (strain X-73) and 3 (strain P-1059) and to determine its passive protectivity against \textit{P. multocida} strains challenge-exposure.

Materials and methods

1. \textit{Bacterial strains and culture media}

All strains used in this present study and their serotypes were shown in Table 1. Bacteria were grown in tryptose broth (TB; Difco, Detroit, MI, USA) at 37°C for 6 h and then cultured on dextrose starch agar (DSA; Difco) at 37°C for 18 h. In order to prepare the immunogen, crude capsular extract (CCE), detergent insoluble fraction of outer membrane protein (DIF-OMP) or major outer membrane protein H (OmpH), 0.2 ml of 6-h bacterial suspension was cultured onto DSA containing 5% sheep blood and then incubated 37°C for 18 h.

2. \textit{Preparation of immunogen}

One single colony was grown in TB at 37°C until OD600 reached 0.1 (approximate \(1 \times 10^8\) cfu/ml). Bacteria were pelleted by centrifugation at 10,000 \(\times\) g for 20 min and washed twice with phosphate-buffered saline (PBS; pH 7.2). Pellets were resuspended in 0.3% formalinized buffer and stored at 4°C until use. In addition, bacterial concentration was determined before the pellets were resuspended. In order to inoculate mice, fixed cells were pelleted by centrifugation at 10,000 \(\times\) g for 20 min and washed twice with PBS (pH 7.2). Then, pellets were mixed with the adjuvant to a final concentration of approximately \(3 \times 10^6\) cfu in 0.5 ml.

| Table 1. Strains of \textit{P. multocida} used in this study |
|---|---|---|
| Strain | Serovar | Origin |
| X-73 | A:1 | Chicken |
| M-1404 | B:2 | Bison |
| P-1059 | A:3 | Turkey |
| P-1662 | A:4 | Turkey |
| P-1702 | A:5 | Turkey |
| P-2192 | A:6 | Chicken |
| P-1997 | A:7 | Herring gull |
| P-1581 | A:8 | Pine siskin |
| P-2095 | A:9 | Turkey |
| P-2100 | A:10 | Turkey |
| P-903 | D:11 | Swine |
| P-1573 | A:12 | Human |
| P-1591 | A:13 | Human |
| P-2225 | A:14 | Cattle |
| P-2237 | D:15 | Turkey |
| P-2723 | A:16 | Turkey |
3. Preparation of CCE

Preparation of CCE was performed by the saline extraction method as described previously. Briefly, bacteria were harvested from 18-h DSA plates and suspended in 2.5% NaCl solution. Bacterial suspensions were incubated at 56°C for 1 h. Then, washed the suspension three times by centrifugation at 17,000 × g for 20 min and final supernatant was dialyzed, concentrated with Carboxymethyl-cellulose sodium salt (Sigma Aldrich, St. Louis, MO, USA) and filtrated through cellulose acetate filter (Toyo Roshi Co. Ltd., Japan). The filtrate was designated as the CCE and determined the protein content by the Bradford's method. CCE was kept at -20°C until use.

4. Preparation of detergent insoluble fraction outer membrane protein (DIF-OMP)

DIF-OMP of strain P-1059 was prepared by the method of Böther et al. Briefly, bacteria on DSA containing 5% sheep blood agar were harvested and then treated with 250 U/ml hyaluronic acid (Sigma) in 10 mM PBS (pH 7.2) at 37°C for 4 h. Then, washed twice with 10 mM PBS (pH 7.2) and resuspended in 50 mM Tris / 1 mM EDTA buffer (pH 8.0). Bacterial cells were disrupted by ultrasonication and large debris was removed by centrifugation at 6,500 × g for 15 min. Supernatant was transferred to new tube and centrifuged at 40,000 × g for in 4°C for 1 h. Pellets, which contain total membrane, was treated twice with 1.5% N-lauryl-sarcosine-sodium-salt (Sigma) in Tris/EDTA buffer. The detergent insoluble fraction was pelleted by centrifugation at 40,000 × g for in 4°C for 1 h and washed once with Tris/EDTA buffer. The final pellets were resuspended in sterile distilled water and kept at -20°C until use. Protein concentration was determined by the Bradford’s method as described previously.

5. Mice immunization for hybridomas production

Seven to eight-week-old BALB/c mice (Funabashi Farm, Funabashi, Japan) were inoculated subcutaneously four times at two week interval. The animal welfare committee of Nippon veterinary and Life Science University controlled use of laboratory animals with the laboratory animal ethics. Experiments were performed in closed system. Experiment rooms and instruments were cleaned with disinfectant for two weeks before and after experiment. Waste products were treated before released to environment.

6. Construction of hybridomas

Hybridomas were produced by fusion the spleen cells of inoculated mice with the P3-X63-Ag8-U1 (P3U1) myeloma cell line with the modified method. The P3U1 cells were grown in growth medium; 0.25 μm-filtered Dulbecco’s Eagles MEM medium (pH 7.2; Nissui, Tokyo,
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Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Irvine Scientific, CA, USA), 200 mM L-glutamine (Gibco, NY, USA), MEM vitamin solution (Gibco), 100 mM MEM sodium pyruvate solution (Gibco) and antibiotic-antimycotic solution (Gibco). Spleen cells of inoculated mice or P3U1 cells were suspended in 10 ml serum-free MEM at 37°C. The cell number were determined with the trypan blue (Sigma) exclusion and mixed spleen cells with P3U1 cells. Mixture was incubated in PEG/DMSO (Sigma) at 37°C for 1 min, serum-free MEM (37°C) for 2 min, 20% FCS-MEM (37°C) for 30 min and pelleted by centrifugation. Then, adjusted the pellets with HAT medium (Gibco) to a final concentration of 1 × 10^6 cells/ml. Cells suspension were transferred (0.15 ml/well) to 96 well cell culture plates (Corning, NY, USA) and incubated at 37°C with 5% CO₂. Cells were fed with 0.1 ml HAT medium after incubated for 3-4 days. Hybrid clones were screened with the supernatant of the clones by enzyme-linked immunosorbent assay (ELISA).

7. Production and purification of cloned antibodies

Monoclonal antibodies were produced by growing the desired antibody hybridomas in growth medium at 37°C with 5% CO₂. Antibodies were also produced in pristine (2, 6, 10, 14-tetramethylpentadecane; Sigma) primed BALB/c mice. Ascitic fluids were collected and antibodies were purified by the Affi-gel protein A (MAP II okit; Bio-Rad) as described by the manufactured instruction.

8. Enzyme-linked immunosorbent assay (ELISA)

Hybridomas supernatant was screened by ELISA. Micro plates (Nunc-immuno™ plate, Denmark) were coated with 10% formalinized whole-cell of *P. multocida* strains in an equal volume of coating buffer; 0.05 M carbonate buffer (pH 9.6), and then incubated at 4°C overnight. Plates were washed three times with saline containing 0.02% Tween 20 (Wako, Osaka, Japan; Saline-T). Non-specific bindings were inhibited with blocking buffer; 5% skim milk (Difco), 0.1% sodium azide (Wako) and PBS containing 0.05% Tween 20 (PBS-T; pH 7.2). Hybridomas supernatant along with diluted positive and negative mouse sera were added to the wells and incubated at room temperature for 1 h. After washed three times with Saline-T, each well was added with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) in blocking buffer and incubated at room temperature for 1 h. Substrate solution containing of 30 mg O-phenylenediamine dihydrochloride (O-PDA; Wako) in 100 ml substrate buffer (0.2 M Na₂HPO₄·12H₂O; 0.1 M C₆H₉O₇; pH 4.8) was added to each well after a further washing and incubated without light at
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room temperature for 30 min. The reaction was stopped by adding of 3 N sulfuric acid (H$_2$SO$_4$; Wako) and absorbance values were observed at wavelength 492 nm with ELISA reader (Immuno Mini NJ 2300, Intermed, Japan). For titration, CCE, DIF-OMP or OmpH-coated plates were reacted with serial two-fold dilutions of mAbs. The end point titer of cell culture supernatant or purified antibodies ascitic fluid was defined as the highest dilution giving and absorbance value greater than 0.7 above background at 492 nm wavelength.

9. Characterization of the epitope

The properties of the epitope recognized by mAbs were tested for its sensitivity to proteinase K treatment $^{10}$ and peroxidate oxidation $^{11}$ by ELISA as described previously. Briefly, overnight CCE or DIF-OMP coated plates were exposed to 200-400 µg/ml of proteinase K in PBS and then incubated at 60°C for 60 min. Following washing, plates were added with mAbs and ELISA was performed as described above. Peroxidate oxidation method was performed by incubated plates with 180 µl/well of PBS-T and stayed at room temperature for 30 min. Plates were washed three times with PBS-T and followed by washing with 50 mM sodium acetate buffer (pH 4.5). Increasing concentration (30-40 mM) of periodic acid in sodium acetate buffer were introduced to each wells and followed by incubation in dark room at room temperature for 1 h. Plates were washed again with sodium acetate buffer and incubated with 50 mM sodium borohydride in PBS at 23°C for 30 min. Then, washed plates three times and followed with described ELISA by employed mAb while PBS was added as the control.

10. Immunoglobulin subclasses

Immunoglobulin isotyping was performed by the mouse monoclonal antibodies K5151 sub-isotyping kit (American Qualex Antibodies, CA, USA) as described by the manufacturer instruction.

11. SDS-PAGE and immunoblotting

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli $^{12}$ and the immunoblot was performed by the method of Towbin et al. $^{13}$. Bacterial suspension from overnight cultures of bacterial strains was pelleted by centrifugation at 15,000 × g for 15 min. Bacterial pellets were adjusted the OD$_{600}$ to 1.0 before resuspended in sample buffer and continuously incubated at 37°C for 2 h. The above suspension was analyzed on a 12.5% polyacrylamide slab gel in a mini-slab apparatus (ATTO Corporation, Tokyo, Japan) and stained with Coomassie blue R-250 (Sigma). Then, proteins were transferred from SDS-PAGE slab gels to nitrocellulose membrane (Amersham Biosciences KK, Tokyo, Japan) and
incubated with a dilution of 1:100 of mAbs and followed to incubate with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). Color development was achieved by incubation with 3, 3-diaminobenzidine (DAB; Sigma) in phosphate-buffered saline (PBS, pH 7.2) and 0.03% H₂O₂.

12. N-terminal amino acid sequence

Bacterial pellets were separated by SDS-PAGE through 12.5% acrylamide slab gels. The proteins were then transferred to immunobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) before probed with the mAb and subsequently subjected to Edman degradation in an applied PPSQ-21/23 protein sequenator (Hitachi, Tokyo, Japan).

13. Passive cross-protection test in mice

Six-week-old female ddY mice (SLC Inc., Hamamatsu, Shizuoka, Japan) were inoculated intraperitoneally with 0.2 ml of purified ascitic fluid of mAb for the experimental group while control mice were inoculated intraperitoneally with ascitic fluid of pristine-treated-BALB/c mice. Four hours after inoculation, experimental or control mice were challenge-exposed intraperitoneally with approximately 100 or 50 LD₅₀ of strain P-1059 or X-73 and then observed for ten days. Necropsy was taken to observe lesion and samples were collected to microbiological laboratory.

Results

1. Production of mAbs and immunoglobulin subclasses

Screening of clones by ELISA for mAb against serotype 3, seven clones showed the OD greater than 2.60 at 492 nm of wave length. Only one clone, designated as a mAb YamaP1, produced antibody against CCE but not react protein in DIF-OMP by ELISA screening. Immunoglobulin isotyping of antibody produced by mAb YamaP1 belonged to IgG₂b.

In addition, three clones; Mame1, Mame2, Mame3, showed the OD greater than 2.60 at 492 nm for mAb against serotype 1 were obtained. Mame1 which showed the highest OD was chosen for this study. The mAb Mame1 also produced antibody against CCE but not react protein in DIF-OMP by ELISA screening. Moreover, immunoglobulin isotyping of antibody produced by mAb YamaP1 belonged to IgG₂b.

2. SDS-PAGE and immunoblotting

Bacterial pellets were probed with mAb YamaP1 by immunoblotting. The result showed a unique reaction with the antigen of approximately molecular weight 39 kDa (Figs. 1 and 2). Strain P-1059 (somatic serotype 3) was immunostained with the mAb YamaP1 while the other 15 reference strains did not (Figs. 1 and 2). In contrast, strain X-73 (somatic serotype 1) was immunostained with the mAb Mame1 while the other 15 reference strains did not (Figs. 2 and 3). The immunoblotting profile indicated that there was no cross-reactivity to the other 15 somatic serotypes.
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Fig. 1. SDS-PAGE of 16 somatic serotypes of *P. multocida* on a 12.5% gel stained with *Coomassie blue*. Lanes: M, molecular mass standards; lanes 1-16, 16 somatic serotypes (Table 1). Numbers on the left indicate the positions of molecular mass standards (in kilo daltons).

Fig. 2. Immunoblot analysis of whole-cell of 16 somatic serotypes of *P. multocida* with Mab YamaP1. Lanes: M, molecular mass standards; lanes 1-16, 16 somatic serotypes (Table 1). Numbers on the left indicate the positions of molecular mass standards (in kilo daltons).
Fig. 3. Immunoblot analysis of whole-cell of 16 somatic serotypes of *P. multocida* with Mab Mame1. Lanes: M, molecular mass standards; lanes 1-16, 16 somatic serotypes (Table 1). Numbers on the left indicate the positions of molecular mass standards (in kilo daltons).

Fig. 4. The effect of proteinase K treatment (left) and periodate oxidation (right) of CCE antigen of strain P-1059 on the binding of mAb YamaP1. The data are presented as the mean OD and the bars indicate standard error of the means.
3. Characterization of epitope

ELISA plates were coated with CCE or DIF-OMP of strains P-1059 or X-73 and treated with a various concentration of proteinase K or periodic acid. Treatment with proteinase K significantly reduced the absorbance value while treatment with periodates had no effect on ELISA means (Figs. 4 and 5)

4. N-terminal amino acid sequence

The results from N terminal analysis showed that mAb YamaP1 recognize the OmpH of strain P-1059 while mAb Mame1 recognize the OmpA of strain X-73.

5. Passive cross-protection test

Both mAbs showed one hundred percents homologous protection (Table 2). Mice showed no clinical sign during ten days post challenge exposure. On the other hand, very low or no protection conferred by both mAbs were observed. Mice were found dead within 24 hrs post challenge exposure. Necropsies showed hemorrhage in internal organs and the bacterial isolation from internal organs showed pure isolates of *P. multocida*.
Table 2. Cross-protectivity conferred by Mabs

<table>
<thead>
<tr>
<th>Mab</th>
<th>Strain of challenge-exposure (cfu/0.1 ml)</th>
<th>No.of survivors/immunization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YamaP1</td>
<td>X-73 (250)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>P-1059 (360)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td></td>
<td>X-73 (125)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>P-1059 (180)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Mame1</td>
<td>X-73 (250)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td></td>
<td>P-1059 (360)</td>
<td>0/5 (0)</td>
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<tr>
<td></td>
<td>X-73 (125)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td></td>
<td>P-1059 (180)</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

Discussion

YamaP1 mAb for strain P-1059 and Mame1 for strain X-73 were generated and were directed against the approximately 35.5 or 37 kDa antigen of the whole-cells. Treatment of Proteinase K to CCE reduced ELISA values inferring that the mAb binding site(s) had been destroyed. On the other hands, treatment of periodic acid to CCE had no effect. This indicated that the epitope in CCE recognized was proteinaceous. The previous studies suggested that the composition of CCE was a LPS-protein complex antigen and the contribution of the CCE to the total antigenicity appeared to be greater for the protein than for carbohydrate (14,15). Moreover, treatment of periodic acid cleaves neighboring hydroxyl groups in sugars without altering the structure of the polypeptide chains (11). The present study, immunoblot analysis of two mAbs with whole-cell obtained from 16 strains of *P. multocida* showed that there is no cross-reactivity among 16 somatic serotypes, which indicated that the mAbs were directed against somatic serotype specific epitope.

The N-terminal analyses indicated the recognized epitopes of two mAbs. Two mAbs recognized the OmpH for strain P-1059 and OmpA for strain X-73, which belongs to the outer membrane protein (Omp) family. The previous studies have developed the mAbs against *P. multocida* strains from the Omp family (16,17,18). Two major outer membrane proteins of *P. multocida*, designated the OmpH and OmpA. The Omp of *P. multocida* was characterized and shown to be related to the porins family and heat-
modifiable protein. Protein H is the major polypeptide of the outer membrane of *Pasteurella multocida*, a bacterium pathogenic for humans and animals. The previous study have suggested that the outer membrane protein always contaminate in the CCE fraction but however the structure of protein is changed due to the extraction by salt and heat. Chevalier et al. have purified this protein to homogeneity by size exclusion chromatography after selective extraction with surfactants and demonstrated its pore-forming ability after reincorporation into planar lipid bilayers. Naturally, the structure of this protein is trimeric arrangement. Remarkably, upon boiling, the trimers were fully dissociated into monomers with an increase of alpha helix and irregular structure, at the expense of beta sheets with the apparent molecular mass of fully denatured monomers ranged between 37 and 41.8 kDa, depending on the electrophoretic system used for analysis. The heat-modifiable 37 kDa protein was famous as the immunostained antigen of many mAb and also showed the acceptable protectivity against challenge-exposure.

Mice infected experimentally with two strains of *P. multocida* were used to study the passive protection test by mAbs. Passive protection test in mice challenge-exposed with strains P-1059 and X-73 showed that there was no cross-protectivity among major strain of avian Pasteurellosis in mice. This suggested that the antibodies induced by a passive immunity in mice prevent colonization of bacteria in internal organs. We conclude that mAb-mediated protection is antigen specific because the protection test was shown only in mice receiving serotype specific mAb and challenged with homologous or heterologous *P. multocida* strains bearing the antigenic determinant recognized by the serotype specific epitope. The previous studies indicated that immunoglobulin mAbs against Omp of *P. multocida* were recognized in protection of mice against lethal challenge infection by means of opsonisation and inhibition of proliferation of *P. multocida*. In our protection study, mice passively immunized with the mAbs against OmpA or OmpH also gave an acceptable homologous protection against strains challenge-exposure.

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References


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*การผลิตโมโนคลอนแอนติบอดีต่อเชื้อ Pasteurella multocida สายพันธุ์ X-73 และ P-1059* 

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ประเทศไทย โมโนคลอนแอนติบอดี (Monoclonal antibody: mAb) ที่เตรียมขึ้นต่อเชื้อสายพันธุ์หลักที่ก่อโรคที่วำดสัตวีปักษ์ ซึ่งผลิตโดยการใช้เครื่องมือทางภูมิคุ้มกัน P-1059 (serovar A:3) หรือ X-73 (serovar A:1) ที่ทำให้หมดฤทธิ์วัคซีนฟอร์มูล้ำให้กับหูไฟฟ้า BALB/c หลังจากนั้นแล้วให้คือ โมโนคลอนแอนติบอดีต่อเชื้อสายพันธุ์ P-1059 จำนวน 3 โคลน (YamaP1-3) และของเชื้อสายพันธุ์ X-73 จำนวน 1 โคลน (Mame1) ตามลำดับ จากผลการวิเคราะห์ด้วยวิธี Immunoblot analysis กับเงื่อนไขมีเจาะ Pasteurella multocida ทั้ง 16 สายพันธุ์ พบว่าไม่โมโนคลอนแอนติบอดีที่ได้จากการเจาะ epitope เดียวกันเรียงสายพันธุ์นั้นๆ การวิเคราะห์ลำดับของกระแสในที่ปลาย N (N-terminal analyses) พบว่า YamaP1-3 จัดจำป้องกัน outer membrane protein (Omp) H ขณะที่ Mame1 จัดจำป้องกัน OmpA ตามลำดับ นอกจากนี้การศึกษาโดยทำย้อมการปฏิกิริยาด้วยแอนติเจน proteinase K หรือตัวบกพร่อง periodic คือ crude capsular extraction (CCE) หรือ detergent insoluble fraction outer membrane protein (DIF-OMP) antigen พบว่า epitope ที่โมโนคลอนจัดจำเป็นสารประกอบจากโพลิไนเตรน ยังไปว่าในนี้สัตว์ที่ทำศึกษาทางทำให้หูไฟฟ้าสถิตมณีกับทาร์จอน (passive immunization) พบว่าโมโนคลอนแอนติบอดีที่ได้จัดจำเข้ามากับการติดเชื้อสายพันธุ์ในหูโครงการ

คำสำคัญ: sdชีซูนิต้า, มิลิติ้งิต้า, โมโนคลอน แอนติบอดี, P-1059, X-73