Production and Characterization of Monoclonal Antibodies against *Actinobacillus (Haemophilus) pleuropneumoniae* Serotype 2

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ABSTRACT. Four hybridoma cell lines producing monoclonal antibodies (MAbs) against *Actinobacillus (Haemophilus) pleuropneumoniae* were established by fusion of mouse myeloma and spleen cells obtained from mice immunized with a serotype 2, strain SH-15. Enzyme-linked immunosorbent assay-inhibition tests with antigens obtained from 12 serotype strains of *A. pleuropneumoniae* and 9 other gram-negative bacteria showed that all the MAbs bound to only serotype 2 strains of *A. pleuropneumoniae*. The epitopes recognized by the MAbs were located on a carbohydrate moiety of lipopolysaccharide (LPS) of the organism, which was sensitive to periodate oxidation. In immunoblotting analyses of LPS obtained from *A. pleuropneumoniae* serotype 2, all the four MAbs reacted specifically with the characteristic ladder bands of LPS detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These results suggest that O-antigen side chains of the LPS are one of the antigenic determinants responsible for the serotype-specificity of *A. pleuropneumoniae*.—**KEY WORDS:** *A. pleuropneumoniae*, LPS, monoclonal antibody.

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Porcine *Haemophilus pleuropneumonia* caused by *Actinobacillus (Haemophilus) pleuropneumoniae* has been one of the major current problems in modern swine industry all over the world [1, 35]. The organism may cause an acute respiratory infection with high morbidity and mortality, or a chronic persistent infection, resulting in general unthriftiness and loss in body weight [11, 42]. Vaccinations against *A. pleuropneumoniae* infection in swine has been attempted with an inactivated vaccine [18, 36]. Eradication of the infection has also been done by eliminating sero-positive animals [38]. For these control measures, it is essential to have information on the serotype(s) prevalent in a given region.

Twelve serotypes of *A. pleuropneumoniae* have been recognized [37]. A wide variety of tests have been developed for serological diagnosis of the disease and serotyping of the isolates, including complement-fixation [8], indirect fluorescent antibody [40, 41], coagglutination [27], indirect

hemagglutination [26], slide agglutination [25], tube agglutination [9], immunodiffusion [7], ring precipitation [25], enzyme-linked immunosorbent assay (ELISA) [34], and latex agglutination [28]. Although these tests are capable of detecting antibodies against serotype-specific antigens, they also detect cross-reacting antigens among serotypes as well [7–9, 34]. Many attempts have been made to analyze the surface antigen structure of *A. pleuropneumoniae* in relation to the serotypes-specificity [7–9, 25–27]. However, the precise cellular locations and characters of the antigens responsible for the serotype-specificity have not been well defined.

Monoclonal antibodies (MAbs) have been used as a valuable tool for analysis of the surface antigen structure of a variety of bacterial cells [5, 10, 30, 32, 39]. In this paper, we report the production and characterization of four MAbs against *A. pleuropneumoniae* serotype 2, the most common serotype in Japan [16, 17, 28]. Using these

MAbs, we have examined the antigen structure of *A. pleuropneumoniae*.

MATERIALS AND METHODS

Bacterial strains and media: The strains used in this study are listed in Tables 2 and 3. Organisms of *A. pleuropneumoniae* and *H. parasuis* grown on S agar plates [19] for 10 hr at 37°C were harvested and suspended in phosphate-buffered saline solution (PBS), pH 7.2, to a concentration of 5×10^{11} cells/ml by spectrophotometry (650 nm, 1×10^{10} cells/ml, absorbance of 0.386) using model 6/20 spectrophotometer (Coleman Instruments Division, Perkin Elmer Corp., Oak Brook, Ill., U. S. A.). Organisms of *Pasteurella multocida* grown on yeast extract-Proteose Peptone (Difco Laboratories, Detroit, Mich., U. S. A.)-cystine agar plates [33] for 6 hr at 37°C were harvested and suspended in PBS by the same manners mentioned above. Each bacterial suspension heated at 56°C for 30 min was employed as an whole cell antigen.

Lipopolysaccharide (LPS): LPS was extracted from serotype 2, strain SH-15 [31] of *A. pleuropneumoniae* with hot 45% phenol and purified by ultracentrifugation [45]. The LPS employed in the present study caused firm gelation in the Limulus amebocyte lysate gel (LAL) test (Limulus single test Wako; Wako Pure Chemical Industries, Tokyo, Japan) at a concentration of 6 ng or higher in dry weight. Protein content of the preparation was less than 1%. Polyacrylamide gel electrophoresis and silver staining results of the LPS (Fig. 2, Lane a) were consistent with the patterns for the LPS obtained from serotype 2, strain 1536 of *A. pleuropneumoniae* by Maudsley *et al.* [24].

Capsular polysaccharide (CP): CP of serotype 2, strain SH-15 of *A. pleuropneumoniae* was purified from the culture supernatant of S broth medium [19] following the method described by Inzana [12].

The CP gave a single precipitation line with antiserum against strain SH-15 in immunodiffusion test. The CP caused firm gelation in the LAL test at a concentration of $> 1 \mu\text{g}$ in dry weight. Protein content of the preparation was less than 1%.

Immunization of mice: Eight-week-old male BALB/c mice (Shizuoka Agricultural Cooperative Association of Laboratory Animals, Hamamatsu, Shizuoka, Japan) were primed intraperitoneally with 0.5 ml of a 1:1 mixture of the whole cell antigen (10^{10} cells/ml) of serotype 2, strain SH-15 of *A. pleuropneumoniae* in $\text{Al}(\text{OH})_3$ -gel (1,500 $\mu\text{g}/\text{ml}$). Two weeks after the injection, the second and the final injections with each 0.1 ml of viable cell suspension in PBS adjusted to ca. 10^{10} colony-forming units/ml were given intravenously at 4-day interval. Three days after the final injection, the mice were bled and their spleens were removed for cell hybridization. A pooled serum sample obtained from the immunized mice was employed as a polyclonal antiserum.

Production of hybridoma and preparation of monoclonal antibodies (MAbs): Spleen cells (10^8 cells) of mice were allowed to fuse, with the aid of 50% polyethylene glycol (Molecular weight, 4,000; Merck & Co., Inc., Darmstadt, West Germany), with myeloma cells, Sp2/0-Ag14 (10^7 cells), maintained in Dulbecco modified Eagle medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum. After washing with the same medium and centrifugation for 5 min at $200 \times g$, 0.1-ml portions of the cell mixture (5×10^5 cells) were placed in wells of a 96-well microplate. Hybrid cells were selected in the same medium containing HAT solution (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 10^{-5} M thymidine) and cloned twice by limiting dilution method [14]. After transplantation of the cloned cells (10^7 cells/mouse) into the peritoneal cavity of the BALB/c mouse primed

with pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis., U. S. A.), the ascitic fluid was obtained. MAb in the ascitic fluid was precipitated with 50% saturated ammonium sulfate and dialyzed against PBS.

Enzyme-linked immunosorbent assay (ELISA): The wells of 96-well assay plates were each coated with 0.1 ml of whole cell antigen (2×10^8 cells/ml) of serotype 2, strain SH-15 of *A. pleuropneumoniae*. After 3 hr at 37°C, the wells were washed five times with PBS containing 0.05% Tween 20. Each well then received 0.2 ml of 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo., U. S. A.). After incubation for overnight at 4°C, the wells were again washed with the same buffer. A sample, 0.1 ml of hybridoma culture supernatant or of diluted ascitic fluid, was added to each well. After 2 hr at 37°C, the wells were washed, and 0.1 ml of peroxidase-conjugated rabbit antibody to mouse immunoglobulin G (Cooper Biomedical Inc., West Chester, Pa., U. S. A.) was added to each well. After 2 hr at 37°C, the wells were washed five times and 0.15 ml of a substrate solution (0.04% o-phenylenediamine in 0.05 M citric acid-0.1 M Na₂HOP₄ buffer, pH 5.0, plus 0.006% H₂O₂) was added. After incubation for 30 min at 37°C, 50 µl of 5 N sulfuric acid was added to stop the reaction. Absorbance at 492 nm was measured with a Titertek Multiscan MCC/340 (Flow Laboratories, Inc., Helsinki, Finland). ELISA titers were expressed as the maximum dilution of each sample showing value exceeding the mean value of the PBS control plus 3-fold of standard deviation.

Treatment of whole cell antigen of serotype 2, strain SH-15 of *A. pleuropneumoniae*: Whole cell antigen (10^9 cells/ml) was heated at 100°C for 15 min or 121°C for 60 min. Treatment with protease was done by incubating with 100 µg/ml of proteinase K (Sigma Chemical Co.) in PBS at 37°C for 3 hr. Periodate oxidation was performed by

incubating with 50 mM of NaIO₄ according to the procedure described by Woodard *et al.* [46].

ELISA-inhibition test: Each MAb diluted at a concentration yielding absorbance value at 492 nm of approximately 1.5 in the ELISA was preincubated for 30 min at 37°C with each of inhibitors, such as whole cell antigens from various bacteria and variously treated or purified antigens of *A. pleuropneumoniae* serotype 2. The incubated samples were centrifuged at $10,000 \times g$ for 10 min at 4°C and the resulting supernatants were tested in ELISA as described above. The percentage of inhibition was calculated by the formula $100(1-A/B)$, where A and B are absorbance at 492 nm in the presence and absence of inhibitor, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting: SDS-PAGE of LPS purified from serotype 2, strain SH-15 of *A. pleuropneumoniae* was performed in a 13% separating gel, using buffer system of Laemmli [20]. Samples were boiled for 2 min with 1% SDS in the presence of 100 mM dithiothreitol. A 8-µl portion of a sample containing ca. 4 µg of carbohydrate was applied. After electrophoresis, the gel was stained by the silver staining procedure [44]. For immunoblotting, the LPS was transferred electrophoretically to nitrocellulose sheets (Toyo Roshi Kaishya, Ltd., Tokyo, Japan) [43]. After blotting, the sheet was soaked overnight in PBS containing 3% BSA, incubated with MAbs for 30 min at room temperature, and washed three times with PBS at 3-min interval. It was then treated for 30 min with peroxidase-conjugated anti-mouse IgG diluted 200-fold with PBS containing 3% BSA. After washing the sheet, the bound peroxidase was allowed to react on 3,3'-diaminobenzidine (0.5 mg/ml)-0.003% H₂O₂ in PBS.

Other methods: The immunoglobulin class, sub-class, and light chain of each MAb

were determined using the Mouse-Typer Sub-Isotyping kit (Bio-Lad Laboratories, Richmond, Ca., U. S. A.). Carbohydrate contents were determined by the method of Dubois *et al.* with D-glucose (Sigma Che-

mical Co.) as the standard [4]. Protein contents were determined by the method of Lowry *et al.* with BSA (Sigma Chemical Co.) as the standard [21].

Table 1. Characteristics of four MAbs and their ELISA titers

Antibodies	Class or subclass	Light chain	ELISA titer ^{a)}
2MAb-1	IgM	k	10 ⁹
2MAb-2	IgA	k	10 ⁹
2MAb-3	IgM	k	10 ⁹
2MAb-5	IgG _{2a}	k	10 ⁸
Polyclonal antiserum ^{b)}	—	—	10 ⁶

a) ELISA was performed in 96-well plates coated with 0.1 ml of the whole cell antigen of serotype 2, strain SH-15. ELISA titers are expressed as the maximum dilution of each sample showing value (A_{492}) exceeding the mean value of the PBS control (0.060) plus 3-fold of standard deviation.

b) Whole cell antigen of serotype 2, strain SH-15 of *A. pleuropneumoniae* was employed as an immunogen. Immunization procedure was described in the text.

Table 2. ELISA-inhibition test of MAbs with various serotype strains of *A. pleuropneumoniae*

Inhibitor ^{a)}	Sero-type	% Inhibition ^{b)}			
		2MAb-1	2MAb-2	2MAb-3	2MAb-5
4074 ^{c)}	1	0	0	0	0
1536 ^{c)}	2	92	73	96	98
SH-15 ³¹	2	95	75	100	100
1421 ^{c)}	3	0	0	0	0
M 62 ^{c)}	4	0	0	0	0
K 17 ^{c)}	5	0	0	0	0
Fomø ^{d)}	6	1	0	0	0
WF 83 ^{d)}	7	0	0	0	0
X 1 ^{e)}	8	0	0	0	0
CVJ 13261 ^{e)}	9	0	0	0	0
D 13039 ^{e)}	10	0	0	5	5
56153 ^{e)}	11	0	0	0	0
8329 ^{e)}	12	0	0	0	0
No inhibitor	—	0	0	0	0

a) Whole cell antigen of each strain was used as an inhibitor at a concentration of 1×10^9 cells/ml.

b) Results of the tests were presented as % inhibition, defined by the formula: $100(1-A/B)$, where A and B are A_{492} in the presence and absence of inhibitor, respectively.

c) Provided by Dr. Nicolet, University of Bern, Switzerland.

d) Provided by Dr. Rosendal, University of Guelph, Canada.

e) Provided by Dr. Nielsen, State Serum Laboratory, Denmark.

Table 3. ELISA-inhibition test of MABs with various gram-negative bacteria

Inhibitor ^{a)}	% Inhibition ^{b)}			
	2MAb-1	2MAb-2	2MAb-3	2MAb-5
<i>Actinobacillus pleuropneumoniae</i>				
biotype 2				
HIM 634 ^{c)}	0	0	0	0
HIM 635 ^{c)}	0	0	0	0
HIM 636 ^{c)}	9	0	0	0
HIM 637 ^{c)}	0	0	0	0
minor group				
202 ^{d)}	0	0	0	0
<i>Haemophilus parasuis</i>				
4800 ^{e)}	0	0	0	0
7441 ^{e)}	0	0	0	0
<i>Pasteurella multocida</i>				
Kobe 6 ^{f)}	0	0	0	0
P-27 ^{f)}	0	0	0	0
<i>A. pleuropneumoniae</i>				
SH-15	88	84	87	79
No inhibitor	0	0	0	0

a) See Table 2, footnote a.

b) See Table 2, footnote b.

c) Provided by Dr. Bertschinger, University of Zurich, Switzerland.

d) Provided by Dr. Rosendal, University of Guelph, Canada.

e) Provided by Dr. Nicolet, University of Bern, Switzerland.

f) Provided by Dr. Isayama, National Institute of Animal Health, Japan.

RESULTS

Characteristics of MABs: Four hybridomas producing MABs against serotype 2, strain SH-15 of *A. pleuropneumoniae* were obtained. The immunoglobulin class, subclass, light chain, and ELISA titer of each MAB are summarized in Table 1.

ELISA-inhibition test of MABs with various serotype strains of *A. pleuropneumoniae* and other gram-negative bacteria: To determine species- and serotype-specificities of the MABs, ELISA-inhibition tests were performed. Significant inhibition of the binding of all the MABs to the coated antigen (serotype 2, strain SH-15) was observed when serotype 2 strains of *A. pleuropneumoniae* were used as inhibitors

(Table 2). Serotype 2, strains SH-15 and 1536 similarly inhibited the binding of the MABs. None of the other serotype strains of *A. pleuropneumoniae* (Table 2) and other gram-negative bacteria tested (Table 3) inhibited the binding of any of the MABs by more than 9%. These results indicate that all the MABs are specific for serotype 2 strains of *A. pleuropneumoniae*.

ELISA-inhibition test of MABs with whole cell antigens of serotype 2, strain SH-15 of *A. pleuropneumoniae* after enzymatic or chemical treatment: Non-treated whole cell antigen (82% to 100%) inhibited the binding of the MABs to the coated antigen (serotype 2, strain SH-15) (Table 4). Also, the whole cell antigen treated with either proteinase K (92% to 100%) or heating at

Table 4. ELISA-inhibition test of MABs with whole cell antigen of serotype 2, strain SH-15 after enzymatic or chemical treatment

Inhibitor ^{a)}	% Inhibition ^{b)}			
	2MAb-1	2MAb-2	2MAb-3	2MAb-5
Whole cell antigen (non-treated)	99	82	84	100
Heat-treated antigen at				
100°C for 60 min	84	70	74	88
121°C for 15 min	78	64	69	82
NaIO ₄ (5 mM) - treated antigen	53	41	49	37
Proteinase K (5 µg/ml)- treated antigen	100	92	99	100
No inhibitor	0	0	0	0

a) Whole cell antigen (1×10^{10} cells/ml) of serotype 2, strain SH-15 was treated, and used as an inhibitor.

b) See Table 2, footnote b.

Fig. 1. ELISA-inhibition test of MABs, 2MAb-1 (○), 2MAb-2 (●), 2MAb-3 (△), and 2MAb-5 (▲), with LPS or CP purified from serotype 2, strain SH-15 of *A. pleuropneumoniae*. Concentrations of inhibitors were shown as carbohydrate content.

100°C (70% to 88%) or 121°C (64% to 82%) inhibited the binding of the MABs. In contrast, the inhibition of the antigen treated with sodium periodate decreased to 37% to 53%, suggesting that the epitopes recognized by the four MABs are located on or close to some carbohydrate moieties.

ELISA-inhibition test and immunoblotting of MABs with LPS or CP purified from serotype 2, strain SH-15 of A. pleurop-

neumoniae: To determine the epitope recognized by the MABs, ELISA-inhibition tests using LPS or CP purified from serotype 2, strain SH-15 of *A. pleuropneumoniae* were performed (Fig. 1). The LPS inhibited the binding of the MABs to the coated antigen, whereas CP did not, even at a concentration of 2,000 µg/ml. The inhibition of the LPS (2,000 µg/ml) treated with sodium periodate (5 mM) decreased to 28% to 20%.

The specificity of the four MABs was further studied by immunoblotting with LPS of serotype 2, strain SH-15. All the MABs reacted specifically with the characteristic ladder bands on the nitrocellulose membrane (Fig. 2, Lanes, b, c, d, and e). These ladder bands of LPS were detected by SDS-PAGE, followed by silver staining (Fig. 2, Lane a).

DISCUSSION

Antisera to an whole cell antigen of certain bacteria contain antibodies against species-specific as well as serotype-specific antigens. Therefore, the use of antiserum against whole cell antigen may lead to erroneous diagnostic results. This prompted us to isolate serotype-specific MABs to be used in serological assays. In this study, we

Fig. 2. SDS-PAGE and immunoblotting analyses of MAb with LPS purified from serotype 2, strain SH-15 of *A. pleuropneumoniae*. Lanes: a, silver-stained LPS; b, c, d, and e, immuno-stained LPS with 2MAb-1, 2MAb-2, 2MAb-3, and 2MAb-5, respectively.

have established four hybridomas producing MAbs against *A. pleuropneumoniae*. All the MAbs were determined to be specific for serotype 2 strains of *A. pleuropneumoniae* as ascertained by ELISA-inhibition tests (Tables 2 and 3). In the competitive binding assays, competitive inhibition was observed among 2MAb-1, 2MAb-2, and 2MAb-3, when 2MAb-5 conjugated to peroxidase was used (data not shown). This suggested that their epitopes were identical or topographically overlapped. These MAbs can be useful tools to analyze the antigen structure and to identify the serotype-specific antigen of *A. pleuropneumoniae*.

In many bacterial species, CP is recognized as the serotype-specific antigen [15]. *A. pleuropneumoniae* serotype 5 CP contained both strain- and serotype-specific antigens based on the ELISA results employing capsular-enriched polysaccharide fraction [6]. The purified CP of *A. pleurop-*

neumoniae serotype 5 was the serotype-specific antigen [12, 13]. Four MAbs reported herein recognized a heat-stable, proteinase K-resistant, and periodate-sensitive antigen. The MAbs were directed against the serotype-specific antigen, as shown in the ELISA-inhibition tests using serotype 1 to 12 strains of *A. pleuropneumoniae*. However, the MAbs failed to react with purified *A. pleuropneumoniae* serotype 2 CP, suggesting that these MAbs recognized serotype-specific antigen other than CP.

Previously, Rosendal *et al.* [40, 41] reported that the most serotype-specific antigen extracted with aqueous phenol was of LPS nature. However, the extract contained antigens that cross-reacted with several serotype strains of *A. pleuropneumoniae*, indicating that some common antigens were also present in the extract. Smooth type LPS of *A. pleuropneumoniae* serotype 5 contained both serotype-specific and cross-reacting antigens, whereas a rough type LPS did not contain detectable serotype-specific antigens but did possess both species-specific and non-species-specific antigens [6]. Enteric bacteria that lose O-side chains from their LPS undergo a smooth-to-rough conversion and lose O-antigen specificity [22]. Organisms of *A. pleuropneumoniae* that underwent a smooth-to-rough conversion lost serotype-specificity [9]. In the present study, we indicated that the serotype 2-specific MAbs recognized the epitope(s) which were located on O-antigen side chains of serotype 2 LPS. Thus, O-antigen side chains of LPS can be one of the antigenic determinants responsible for the serotype-specificity of *A. pleuropneumoniae*. Further studies are necessary to determine the reactivity of the MAbs with LPSs purified from serotype 1 to 12 strains other than serotype 2 and to identify the precise structure of epitope(s) on O-side chains exhibiting serotype-specificity.

LPS from gram-negative bacteria have

been reported to be one of the virulence factors and to influence neutrophil functions [3, 23, 29]. LPS of *A. pleuropneumoniae* are also implicated in the pathogenesis of necrohemorrhagic lesions in peracute pneumonia [2, 11]. However, it is unknown whether antibodies directed to LPS or the antigenical polysaccharide chains enhance resistance to *A. pleuropneumoniae* infection. The MAbs obtained in the present study, which were directed against LPS of *A. pleuropneumoniae* serotype 2, could be helpful to characterize the role of LSP in the porcine *Haemophilus pleuropneumoniae*. Studies on protective effects of the MAbs to *A. pleuropneumoniae* infection are in progress.

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要 約

Actinobacillus (Haemophilus) pleuropneumoniae 血清型 2・菌株に対するモノクローナル抗体の作製とその性状：中井豊次・堀口安彦・久米勝巳(北里研究所附属家畜衛生研究所)——*A. pleuropneumoniae* に対する 4 クローンのモノクローナル抗体(MAb)を常法により作製し、それらの性状を解析した。ELISA—阻止試験の結果から、4 クローンの MAb はいずれも *A. pleuropneumoniae* 血清型 2・菌株とのみ反応し、LPS の NaIO_4 感受性抗原部分を特異的に認識すると考えられた。SDS-PAGE とイムノブロッティングにより、これらの MAb はすべて血清型 2・菌株 LPS の O-抗原側鎖部分と反応することがわかった。以上の成績から、LPS の O-抗原側鎖は *A. pleuropneumoniae* の血清型特異性に関与する抗原のひとつであることが示唆された。