Effect of Passive Immunization with Serotype-Specific Monoclonal Antibodies on *Actinobacillus pleuropneumoniae* Infection of Mice

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ABSTRACT. Specific monoclonal antibodies (MoAbs) to *Actinobacillus pleuropneumoniae* (APP) serotypes 1 and 2 which recognized serotype-specific antigens were produced. It was revealed that the two serotype-specific MoAbs H1–18 and H22–7 recognized O polysaccharides of the lipopolysaccharide (LPS) from APP serotypes 1 and 2, respectively, in the results of antigen analysis by means of SDS-PAGE and Western blotting. Furthermore, ddY mice immunized passively with the above type-specific MoAbs were protected against challenge infection by the homologous serotype of APP at 24 or 48 hr later. However, H1–9 and H3–2 MoAbs recognizing serotype-common protein antigens of APP did not show signs of any protective effect. These results showed that LPS from APP bacterial cells was one of the structural substances in the serotype-specific antigens, and an important component as one of the antigens protecting against the homologous serotype strain.—KEY WORDS: *Actinobacillus pleuropneumoiae*, LPS, monoclonal antibody, passive immunization, protective antigen.

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Inactivated and adjuvanted whole cell bacterin of the serotypes 2 and 5 had been developed already and applied for the prevention of swine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (APP) in Japan. However, these bacterins are not only serotype-specific but also do not have a sufficient protective effect [11, 12]. It is therefore very important and useful to develop a more effective vaccine, and more sensitive and specific diagnostic kits for the identification and serotyping of APP isolated from field specimens.

Since the technique of cell fusion was applied to immunoglobulin production by Köhler and Milstein [10] in 1975, the monoclonal antibody (MoAb) has been rapidly applied to wide field of medical science and biology. Various kinds of monoclonal antibodies (MoAbs) to APP already prepared by many researchers were applied to analyze the serotype expressible substances, to immunohistochemical staining for lesions and to search for virulence factors in bacteria [8, 15, 16, 23].

The purpose of this study is to analyze the relationship between serotype expressible substances and protective antigens of APP. We attempted to produce serotype-specific MoAbs to APP serotype 1 and 2 as the first step in screening to detect protective antigen components from APP whole cells, and analyzed the specificity and antigenic epitopes of MoAbs. The protective effects of these MoAbs were examined in passive immunity to the homologous and heterologous serotype strains of APP in mice.

MATERIALS AND METHODS

APP strains and medium: The following strains were used for the preparation of antigens for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis, i.e. serotype 1: Shope 4074 and HA-337, serotype 2: SHP-1 and 720, serotype 3: S 1421, serotype 4: M 62, serotype 5a: K 17, serotype 5b: L 20, serotype 6: Fem ϕ, serotype 7: WF 83, serotype 8: CCM 3803, serotype 9: CVJ-13261, serotype 10: D-13039, serotype 11: 56153 and serotype 12: 1096. Of these strains, strain HA-337 and strain 720 were used for the production of MoAbs to serotypes 1 and 2.

Chicken meat infusion agar medium was used for the preparation of each antigen for the harvesting of sufficient bacterial growth. The composition of the medium was as follows: 50% chicken meat infusion, 1% polypepton-S (Wako Pure Chemical Industries, Ltd., Japan), 0.5% NaCl, 0.01% β-nicotinamide-adenine dinucleotide (β-NAD; Sigma Chemical Co., U.S.A.) and 1.3% agar (Bitek^{TM}Agar; Difco Laboratories, U.S.A.).

Preparation of challenge inoculum: Bacteria used in the challenge inoculation in passive immunization tests grown in heart infusion broth (HI broth; Eiken Chemical Co., Ltd., Japan) supplemented with 5% chicken serum (GIBCO Life Technologies, Inc., U.S.A.) and 0.001% β-NAD for 6 hr at 37°C were harvested by centrifugation (10,000 × g, 10 min) and each challenge dose prepared with HI broth and 10% mucin (Difco) solution.

Production of MoAbs: Antigens for production of the MoAbs were prepared according to the method of Nakai *et al.* [15]. Six 5-week-old female BALB/c mice (Japan SLC Co., Ltd., Japan) were immunized five times intraperitoneally with 10 μg of the above antigen of the HA-337 strain (serotype 1) emulsified in incomplete Freund's adjuvant at two week intervals. Three days after the final immunization, spleen cells removed from an immunized mouse were fused with X63-Ag8-6.5.3. myeloma cells using 50% (W/V) polyethyleneglycol 1,000 (Wako) as the fusogenic agent. Screening for anti-APP

serotype 1 immunoglobulins (Igs) was carried out by the indirect enzyme-linked immunosorbent assay (ELISA) with inactivated antigens. Colonies were subcloned three times by the limiting dilution method. Hybridomas producing specific MoAb to APP serotype 2 were established in a manner similar to the above method, using APP serotype 2 strain 720 as antigens. On the other hand, two kinds of hybridomas producing common MoAbs to APP serotypes 1 to 12 were also established during screening of the APP serotype 1 specific MoAbs. All hybridomas producing MoAbs ($0.5–1\times10^7$ cells) to APP serotypes 1 and 2 were transplanted intraperitoneally into the BALB/c mice, and each MoAb in the ascitic fluid was highly purified on a Protein A Sepharose column (Pharmacia LKB Biotechnology, Sweden).

The Ig class and subclass of each MoAb were determined with the Mouse-Typer Kit (Bio-Rad Laboratories, Richmond, U.S.A.).

SDS-PAGE and immunoblotting: SDS-PAGE for the analysis of antigens was performed in 5% stacking and 14% separating gels by the method of Laemmli [13].

Immunoblotting was performed for the analysis of antigens and MoAbs by the method of Towbin *et al.* [24]. After electrophoresis, the gel was transferred onto nitrocellulose membrane (Bio-Rad) and incubated with 50 m*l* of PBS containing 4% (w/v) skim milk (Difco) for 1 hr. The membrane was suspended in 0.15 M phosphate-buffered saline (PBS, pH 7.4) containing 0.05% (v/v) Tween 20 (Tw 20-PBS) and washed three times with the same buffer at 5 min intervals. The membrane was soaked with 15 m*l* of hybridoma culture supernatant, and washed three times with the above-mentioned buffer after being incubated for 1 hr. Subsequent immunoblotting procedures were carried out with the Avidin-Biotinylated Horse-radish Peroxidase Complex (ABC) Kit (Vector Laboratories Inc., U.S.A.) [5]. All steps were done at room temperature.

Enzyme and chemical treatments of the whole cell antigen: In order to analyze the antigen epitopes recognized by MoAbs, whole cell antigen ($10^8–10^9$ cells/m*l*) of each serotype was treated with Proteinase K (Sigma) or Glycosidases "Mixed" (Biochemicals Co., Japan) (100 μg/m*l*, 37°C, 3–5 hr). Periodate oxidation was carried out for 12–24 hr at 4°C with 50 mM of $NaIO_4$ (Nakarai Chemicals Ltd., Japan), and then the reactivity of antigens to MoAbs were analyzed by immunoblotting.

Purification of the structural antigens: Capsular polysaccharide (CP) and lipopolysaccharide (LPS) were purified from the lyophilyzed cells of APP serotypes 1 and 2 as described by Fenwick and Osburn [3], and Westphal and Jahn [26], respectively. LPS O polysaccharide, LPS core oligosaccharide and lipid A were purified by the method of Byrd and Kadis [1], and Inzana *et al.* [9]. The purified antigens from bacterial cells except LPS were examined for their reactivity to serotype-specific MoAbs of APP by sandwich ELISA.

Indirect ELISA: Each well in the microtiter plates coated with 100 μl of whole cell antigen of serotypes 1 to 12 of APP ($1–5\times10^8$ cells/m*l* of 10 mM citrate buffer, pH 9.0) was immobilized for 1 hr at 37°C. The wells were then washed with Tw 20-PBS, and 100 μl of PBS containing 1% (w/v) bovine serum albumin (BSA Fraction V; Sigma) (1% BSA-PBS) was added, and incubated for 1 hr at 37°C. They were washed again with Tw 20-PBS, and 50 μl of culture supernatant of hybrid cells was added. After being incubated for 1 hr at 37°C, the wells were washed five times with Tw 20-PBS. Fifty μl of a horseradish peroxidase-conjugated secondary antibody in 1% BSA-PBS was added to each well and reacted for 1 hr at 37°C. They were washed five times again with the same buffer, and 50 μl of the substrate solution [0.0675% (w/v) 2, 2'-AZINO-bis (3-ETHYLBENZTHIAZOLINE-6-SULFONICACID) Diammonium Salt; Sigma, 0.025% (v/v) H_2O_2 in 100 mM citrate buffer, pH 4.0] was added. After incubation for 10 min at 37°C, 50 μl of 0.32% NaF solution was added to stop the reaction. The change in absorbance was observed at 414 nm with an EIA reader (Bio-Rad).

Sandwich ELISA: The microtiter plates were coated with-IgG (about 1 μg) of the serotype-specific MoAbs of serotype 1 or 2. Then, each well was added 100 μl of 1% BSA-PBS. After incubation for 1 hr at 37°C, these wells were washed with Tw 20-PBS. Fifty μl of serial two-fold diluted the purified antigens (500 μg/m*l* in 1% acetic acid) were added to each well. After incubation for 1 hr at 37°C, the wells were washed 5 times with Tw 20-PBS. Fifty μl of a horseradish peroxidase-conjugated serotype-specific MoAb in 1% BSA-PBS was added to each well for 1 hr at 37°C. Subsequent ELISA steps were carried out as described above.

Protective effect of MoAbs: The protective effect of the MoAbs was evaluated by two passive immunization experiments in mice.

Immunized groups consisting of five 5-week-old female ddY mice were each injected intravenously, intraperitoneally or subcutaneously with 100 μg/dose of H1–18 or H22–7 MoAbs. The immunized mice were challenged intraperitoneally with ten LD_{50} of bacterial suspension of APP serotype 1 or 2 at 24 or 48 hr after immunization.

The cross immunity of H1–18 and H22–7 MoAbs and the protective effect of H1–9 and H3–2 MoAbs were also evaluated in mice. The constitution of the experimental groups and immunizing dose of the four MoAbs were the same as above. Each group except the control was immunized intravenously with each MoAb. They were challenged intraperitoneally with ten, three and one LD_{50} of bacterial suspension of APP serotype 1 or 2 at 24 hr after immunization.

The LD_{50} of Shope 4074 (serotype 1) and SHP-1 (serotype 2) in ddY mice were calculated to be 4×10^5 CFU/0.5 m*l* and 1.3×10^6 CFU/0.5 m*l* by the method of Reed and Muench [19].

RESULTS

Characteristics of the MoAbs: A total of fifteen hybrido-

mas producing MoAbs to APP (nine hybridomas producing type-specific MoAbs to serotype 1, four hybridomas producing type-specific MoAbs to serotype 2 and two hybridomas producing common MoAbs to serotypes 1 to 12) were established. In this study, four of fifteen MoAbs which exhibiting high reactivity were selected and used in subsequent experiments. MoAbs H1–18 and H22–7 were identified to be serotype-specific MoAbs, and a subclass of Igs of their MoAbs were confirmed to be IgG_{2a} in H1–18 and IgG_3 in H22–7. Antigen epitopes which recognized H1–18 and H22–7 MoAbs were sensitized by means of periodate oxidation and glycosidase treatments. These two MoAbs reacted strongly only to LPS O polysaccharide, but only slightly to CP, LPS core oligosaccharide and lipid A. On the other hand, MoAbs H1–9 and H3–2 with IgG_{2a} as a subclass of Igs reacted to common antigens of serotypes 1 to 12. These two MoAbs recognized antigens which reacted sensitively to protease treatment (Table 1).

SDS-PAGE and immunoblotting: The whole cell antigens of APP serotypes 1 to 12 were analyzed by SDS-PAGE, and then their protein and carbohydrate put onto a nitrocellulose membrane were analyzed by the MoAbs immunoblotting method. MoAbs H1–18 and H22–7 were reacted strongly only to the homologous serotype antigen, and their broad and ladder bands, which is estimated approximately more than 106 Kd to less than 40 Kd in molecular weight, were detected (Fig. 1-A and B). On the other hand, MoAbs H1–9 and H3–2 commonly to serotypes 1 to 12, and the molecular weight of bands detected by MoAb H1–9 was estimated to be 80 Kd, and several bands detected by MoAb H3–2 were up to around 50 Kd (Fig. 1-C and D).

Protective effect of MoAbs in mice: As shown in Table 2, three groups of mice injected intravenously, intraperitoneally and subcutaneously with MoAb H1–18 and H22–7 were protected completely against a challenge with

Table 1. Characteristics of the monoclonal antibodies to *A. pleuropneumoniae*

Monoclonal antibody	Immunoglobulin subclass	Serotype specificity	Antigenic sensitivity	Determinant of antigen epitope
H1–18	IgG_{2a}	1	Glycosidase, $NaIO_4$	LPS O polysaccharide
H22–7	IgG_3	2	Glycosidase, $NaIO_4$	LPS O polysaccharide
H1–9	IgG_{2a}	1 to 12	Protease	N.D.[a)]
H3–2	IgG_{2a}	1 to 12	Protease	N.D.

a) Not detectable.

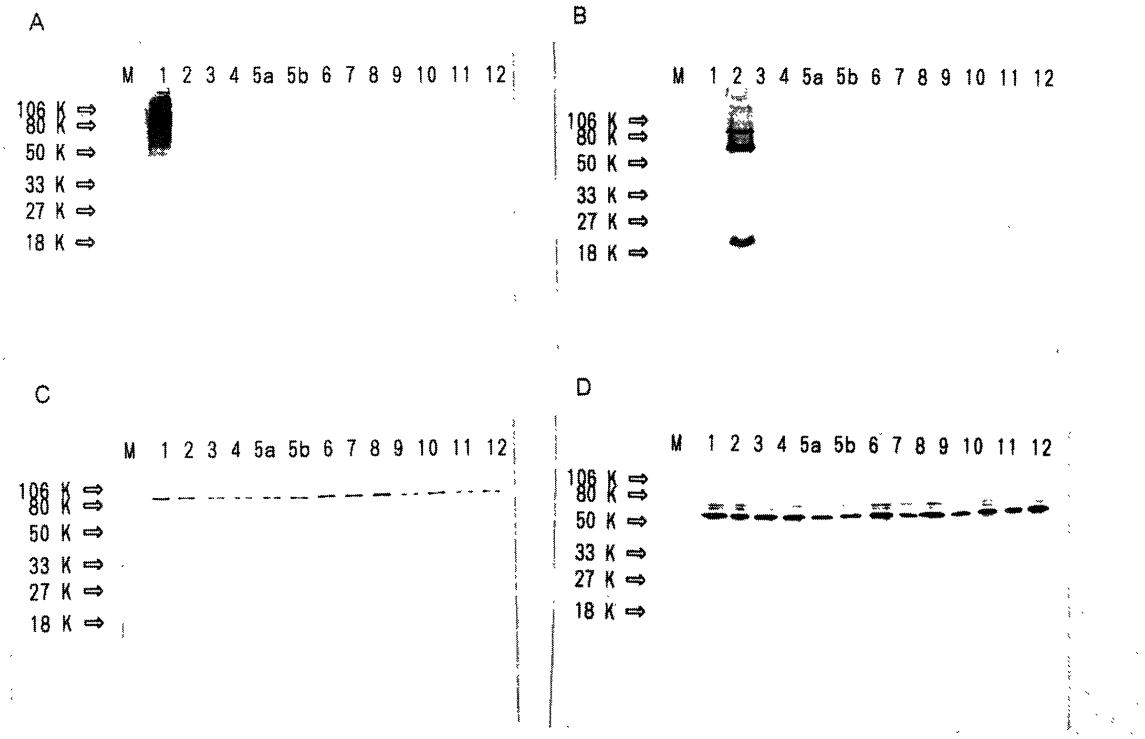

Fig. 1. Western blotting analysis of whole cell antigens probed with monoclonal antibodies.
M: molecular weight standards, lanes 1 to 12: whole cell antigens of APP serotypes 1 to 12. A: with serotype 1-specific MoAb H1–18, B: with serotype 2-specific MoAb H22–7, C: with serotypes 1 to 12 common MoAb H1–9 and D: with common MoAb H3–2.

Table 2. Protection of mice immunized passively with the serotype-specific monoclonal antibodies to challenge by *A. pleuropneumoniae* serotype 1 or 2

Group	Monoclonal antibody	Route of inoculation	Challenge strain with 10 LD_{50}[a)]	Time (hr) at challenge after immunization	
				24	48
Immunized	H1–18	Intravenous	Shope 4074	0[b)]	0
		Intraperitoneal	(serotype 1)	0	0
		Subcutaneous		0	0
Non-immunized control				5	5
Immunized	H22–7	Intravenous	SHP-1	0	0
		Intraperitoneal	(serotype 2)	0	0
		Subcutaneous		0	0
Non-immunized control				5	5

Mice in each group were immunized with 100 μg/0.2 m*l* of each monoclonal antibody via three routes of inoculation. They were challenged intraperitoneally at 24 hr or 48 hr after immunization.
a) One LD_{50} of Shope 4074 and SHP-1 were 4×10^6 CFU/0.5 m*l* and 1.3×10^7 CFU/0.5 m*l*, respectively.
b) Number of dead mice out of 5 mice examined.

Table 3. Cross protection of mice immunized passively with the serotype-specific and common monoclonal antibodies to challenge by *A. pleuropneumoniae* serotype 1 or 2

Group	Monoclonal antibody	Challenge strain					
		Shope 4074 (serotype 1)			SHP-1 (serotype 2)		
		10 LD_{50}[a)]	3 LD_{50}	1 LD_{50}	10 LD_{50}	3 LD_{50}	1 LD_{50}
Immunized	H1–18	0[b)]	1	0	3	5	4
	H22–7	4	5	2	1	0	0
	H1–9	5	3	3	5	4	4
	H3–2	5	5	3	4	5	3
Non-immunized control		5	4	5	4	5	5

Mice in each group were immunized intravenously with 100 μg/0.2 m*l* of each monoclonal antibody. They were challenged intraperitoneally at 24 hr after immunization.
a) Challenge dose. Refer to the footnote in Table 2.
b) Number of dead mice out of 5 mice examined.

10 LD_{50} of the homologous strain Shope 4074 or SHP-1 at 24 or 48 hr after passive immunization through each route. On the other hand, all of two control groups without immunization were died within 24 hr after the challenge. The cross protective effect of these MoAbs H1–18 and H22–7 in mice each injected intravenously with 100 μg/0.2 m*l* were evaluated with serotype 1 strain Shope 4074 and/or serotype 2 strain SHP-1. A significant protective effect in mice immunized with MoAbs H1–18 and H22–7 was revealed only against the homologous serotype strain, but not revealed against the heterologous one, even when a lower dose of the APP strain was challenged. On the other hand, the MoAbs H1–9 and H3–2 recognizing a common serotype antigen of each serotype 1 to 12 of APP did not show signs of any protective effect (Table 3). Almost all of each group treated with above MoAbs died within 24 hr after the challenge as well as the controls.

DISCUSSION

The difference of serotype in APP has been reported to be based on the carbohydrate structure of CP [4, 7, 14] or O antigen polysaccharides of LPS [15, 20, 21]. Perry *et al.* found that the difference in the structure of the serotypes was caused in the carbohydrate chain of CP and LPS, in serotypes 1 to 12 of APP by chemical and nuclear magnetic resonance methods [17]. The protective antigens of APP have been reported to relate to outer membrane proteins [18], CP [6, 7] and LPS [3] as intracellular antigens, and recently to hemolysin [2] and cytotoxin [22] as extracellular antigens.

In this study, the characteristics of four MoAbs which are recognized to be type-specific antigens of APP serotype 1 or 2 and common antigens in APP serotypes 1 to 12 were analysed, and the passive immunizing effects of these MoAbs were evaluated in mice in order to confirm the protective antigens in APP cells. Antigen epitopes

recognizing serotype-specific MoAbs H1–18 or H22–7 were suggested to be polysaccharides composed of APP serotypes from the results of analysis of the antigens by SDS-PAGE, immunoblotting and sensitivity to glycosidase and $NaIO_4$ treatments. Furthermore, from the results of the reactivity of serotype-specific MoAbs to CP, LPS O polysaccharide, LPS core oligosaccharide and lipid A of LPS composed of APP serotype 1 or 2, they were recognized as polysaccharides in O antigen of LPS from each serotype.

On a role of the pathogenesis of APP in swine pleuropneumonia, Udeze *et al.* [25] examined histopathologically lung lesions performed experimentally-intranasally to CF1 mice and intratracheally to pigs-with intact APP serotype 1 cells and their purified LPS. According to their data, a LPS endotoxin and a heat-labile factor may be involved in the pulmonary lesion development in the acute phase of swine pleuropneumonia. In this study, the intraperitoneal route was used as the challenge route for mice immunized with MoAbs. It might not be necessarily the most suitable route for the challenge of APP infection in mice, since this route is not a natural infection route for these bacteria in pigs. However, it was used only as a screening test for the evaluation of the protective effect of a component antigen of APP and its MoAbs in mice.

Inzana *et al.* [8] reported that pigs immunized passively with serum from pigs which recovered from the experimental lethal infection by serotype 5 of APP showed protection against a challenge with the homologous strain. However, porcine antiserum immunized with CP of APP serotype 5 did not have a protective effect even in challenging the homologous serotype strain of APP. It was suggested that at least the antibodies to the capsule of APP serotype 5 was not enough protective against APP infection.

Smith and Lida [23] verified that pigs injected intraperitoneally and/or intranasally with mouse MoAbs to APP serotypes 1 and 3 showed signs of a successful protective effect against experimental challenge with the homologous serotype only, and were not protected against the heterologous type. Furthermore, protection against the experimental infection with APP was somewhat less in other piglets to which MoAb was given only by the intranasal route 1 hr before the organism was administered than in those given the MoAb intraperitoneally 24 hr beforehand. Our serotype-specific MoAbs H1–18 and H22–7 revealed a sufficiently protective effect against challenge infection by its respective homologous serotype at 24 or 48 hr after passive immunization through an intravenous, intraperitoneal or subcutaneous route. MoAbs H1–9 and H3–2 which recognized APP common structural proteins did not protected against any other serotypes of APP. It was suggested that intracellular protein found commonly in each serotype and unrelated to the expression of the serotype could not be an effective protective antigen. Conversely, it was suggested too, that antigen epitopes which recognized two serotype-specific MoAbs such as H1–18 and H22–7 were not only the serotype-specific but also one of the components of a protective antigen in the homologous serotype. These MoAbs would be an important tool for the serotyping of APP isolated from field materials, and for the screening and purification of protective components from APP bacterial cells.

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