Quantitative Diversity of 67 kDa Protective Antigen among Serovar 2 Strains of *Erysipelothrix rhusiopathiae* and Its Implication in Protective Immune Response

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ABSTRACT. Mouse monoclonal antibodies (MAbs), raised against the NaOH-extracted antigen of *Erysipelothrix rhusiopathiae* strain Kyoto (serovar 2), recognized two different epitopes on a single protein of molecular weight 67 kDa. The MAbs were classified as protective or non-protective against strain Fujisawa (serovar 1). In immunoblotting analysis using the MAbs, fifteen wild strains were shown to contain different amounts of 67 kDa protective antigen. Each formalin-killed whole cell vaccine (bacterin) prepared from the fifteen wild strains conferred different levels of protection against strain Fujisawa in mice. Bacterins prepared from wild strains with larger amounts of 67 kDa protective antigen tended to give high levels of antigen-specific antibody and better protection to mice. These results indicate that the amount of 67 kDa protective antigen which influences the induction of protective immune responses may vary substantially among the strains of *E. rhusiopathiae* (serovar 2).

KEY WORDS: bacterin, *Erysipelothrix rhusiopathiae*, monoclonal antibody, 67 kDa protective antigen.

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Erysipelothrix rhusiopathiae (*E. rhusiopathiae*) is a Gram-positive bacillus that causes erysipelas in mammals, birds and fishes [1, 2, 10, 13]. In swine, erysipelas may occur as a septicemic disease with high mortality in an acute form, and arthritis or endocarditis in a chronic form [22]. The disease is important because of its economic impact on the pig industry. In Japan, a live vaccine prepared from an acriflavine-fast attenuated strain Koganei 65-0.15 has been used widely to prevent swine erysipelas [8, 12, 17], and a whole cell bacterin was also developed recently. In other countries, a bacterin as formalin-killed whole cell vaccine has been used [21]. These vaccines induce a high level of antibody against the 66–64 kDa protein of the bacteria. In a previous study, we reported that the NaOH-extracted antigen also induced the production of antibody against the protein in swine [5]. Neither qualitative nor quantitative variation in the surface protective antigen of the bacteria (serovar 2) was demonstrated [23]. However, there is another report which describes that low or moderate virulent strains were less reactive to the 66–64 kDa antigen, suggesting that the amount of the antigen might be directly or indirectly related to virulence [14]. The diversities of the 67–64 kDa protective antigen of *E. rhusiopathiae* strains have not been investigated in detail, and the aim of this study was to characterize quantitatively the 67–64 kDa protective antigen of the bacteria (serovar 2) in relation to induction of protective immune responses. To achieve this aim, immunoblotting analysis and protection tests using monoclonal antibodies (MAbs) and bacterins prepared from various strains, were performed.

MATERIALS AND METHODS

Bacterial strains: Strain Kyoto (serovar 2) of *E. rhusiopathiae* was used to prepare mouse MAbs. Virulent strain

Fujisawa (serovar 1a) was used for challenge exposure. The eleven wild strains and four reference strains (serovar 2) used in this study are shown in Table 2. They were obtained from the National Veterinary Assay Laboratory, Kokubunji, Tokyo, Japan.

Preparation of mouse MAbs: The NaOH-extracted antigen was prepared as described previously [5]. The antigen was emulsified with an equal volume of Freund's incomplete adjuvant (Difco, Detroit Michigan, U.S.A.), and the immunogen was injected twice intramuscularly into 5-week-old BALB/c female mice at 2-week intervals. On day three before fusion, 0.2 ml of the NaOH-extracted antigen was injected intraperitoneally into the immunized mice. The spleen cells removed from the mice were fused with mouse myeloma cells (SP2/0-Ag14) in 50% polyethylene glycol 4000 (Merck & Co., Inc., Darmstadt, Germany) according to the method of Kohler and Millstein [6]. Culture supernatants from hybridomas were tested for the presence of antibody directed against *E. rhusiopathiae* by enzyme-linked immunosorbent assay (ELISA). The positive hybridoma cells were cloned by the limiting dilution method. These cells were injected into pristane (Sigma, St. Louis, U.S.A.)—primed BALB/c female mice to obtain ascites fluid. MAbs recognizing 67 kDa protective antigen were screened by immunoblotting analysis (Fig. 1). The immunoglobulin subclass of the MAbs was determined with a mouse monoclonal antibody isotyping kit (Amersham Japan, Tokyo, Japan). To perform passive immunization, MAbs were purified from the ascites fluid by protein A column chromatography (Bio-Rad, Richmond, CA., U.S.A.).

Passive immunization with MAbs: Six groups of ten 5-week-old ddY female mice (Japan SLC Inc., Shizuoka, Japan) were injected intraperitoneally with 200 µg/dose of a different purified MAbs. Ten mice in the control group were

inoculated with 0.5 ml of the normal mouse serum. Four hours later, all the mice were challenge-exposed by a subcutaneous injection of 3.5×10^3 colony forming units (CFU) of strain Fujisawa. The mice were observed for 10 days after the challenge exposure, and their rate of survival was recorded.

Preparation of bacterins: *E. rhusiopathiae* strains were cultured in trypticase soy broth (TSB, Becton Dickinson, U.S.A.) containing 0.1% Tween 80 and 2% horse serum (TS-TSB) at 37°C for 24 hr. The bacteria were inactivated by 0.3% (v/v) formalin and harvested by centrifugation at $10,000 \times g$ at 4°C for 20 min. The bacterial cells were washed with phosphate-buffered saline (PBS), and then resuspended to a concentration of an optical density (OD) of 1.0 at a wavelength of 420 nm.

Assessment of protective efficacy of bacterins: Mice were randomly divided into 16 groups of 10 animals. Fifteen groups of the mice were injected intramuscularly with 0.1 ml of the bacterin twice at 2-week intervals, and the remaining group was kept as a non-immunized control. On day 14 after the second injection, 5 mice of each group were challenge exposed by subcutaneous inoculation with 3.0×10^3 CFU of strain Fujisawa. The mice were observed for 10 days after the challenge-exposure, and their mortality was recorded. To measure antibody titers against 67 kDa antigen the remaining 5 mice were bled on the day of the challenge.

Antibody assay: The serum antibody titer against 67 kDa antigen was measured by a competitive ELISA. One hundred microliters of the NaOH-extracted antigen diluted with carbohydrate buffer, pH 9.6 was added to every well of an ELISA plate. After incubation at 4°C for overnight, the plate was washed with PBS containing 0.05% Tween 20 (T-PBS). One hundred microliters of serum sample in dilution of 100 was added to each well. After incubation at 37°C for 1 hr, the plate was washed with T-PBS, 100 μ l of biotin-labeled MAb Kn-L diluted 1:16,000 was added to every well, and then incubated at 37°C for 1 hr. After washing, 100 μ l of peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories, Inc, U.S.A.) diluted 1:10,000 was added to every well, and the plate was incubated at 37°C for 1 hr. After washing, O-phenylene diamine (SIGMA, U.S.A.) substrate was added to every well and the plates were incubated at 30°C for 30 min. Fifty microliters of 1 M H_2SO_4 were added into all wells to stop the reaction, and the OD was read at a wavelength of 490 nm (OD_{490}). ELISA antibody titer was shown as the ratio of inhibition, which was calculated as: $100 \times (OD_{490} \text{ in the absence of mice serum} - OD_{490} \text{ in the presence of mice serum}) / OD_{490} \text{ in the absence of mice serum}$.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting: *E. rhusiopathiae* strains grown in TS-TSB at 37°C for 24 hr were harvested by centrifugation at $10,000 \times g$ at 4°C for 20 min. The pellet was washed 3 times with PBS, and resuspended to a concentration of 1.0 at a wavelength of 420 nm. The bacterial cells were solubilized in SDS-PAGE sample buffer composed of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.03% bromophenol blue and 0.063 M Tris-HCl buffer, pH 6.8, by heating in boil-

ing water for 3 min. The samples were electrophoresed on 12% gel by the method of Laemmli [7]. The separated proteins were transferred to Immobilon membranes (Millipore, Bedford, MA., U.S.A.) according to the method of Towbin *et al.* [15]. The membranes or strips were blocked with 5% gelatin in PBS at room temperature for 1 hr, washed three times with T-PBS and then incubated with purified MAbs at room temperature for 1 hr. They were washed and incubated with peroxidase-conjugated anti-mouse IgG (CAPPEL: U.S.A.) in dilution of 2,000 with PBS at room temperature for 1 hr. After washing three times, the protein bands were visualized with 3-3'-diaminobenzidine and 0.02% H_2O_2 .

RESULTS

Isotype and protective ability of MAbs: Six hybridomas producing MAbs which recognized 67 kDa protein in the NaOH-extracted antigen were obtained and classified into two groups (group A: Kn-L, E, D and F; group B: Kn-Q and R) by competitive binding assay (data not shown). The subclasses of Igs of these MAbs were determined as IgG1 (group A) and IgG2b (group B). In immunoblotting analysis, all the MAbs reacted with the 67 kDa protein of single band (Fig. 1). MAbs of group B showed stronger reactivity than those of group A.

In the passive immunization test, all the MAbs of group A had a protective effect (80 to 100% protection) against infection with strain Fujisawa in mice (Table 1). On the other hand, MAbs of group B and normal mouse serum did not provide protection.

Immunoblotting analysis of the 67 kDa protective antigen of various strains: The results of immunoblotting analysis using MAb Kn-L of group A are shown in Fig. 2. The intensity of the 67 kDa protein band varied widely among the *E. rhusiopathiae* strains. The protein bands of strains Shizuoka-63, 82-510, Saitama-1, CN3461 and SE-9 were strongly stained. In contrast, the protein bands of strains 82-482, 82-930, T199, 44 and AN-4 were weakly stained. MAb Kn-Q of

Fig. 1. Immunoblotting analysis of the 67 kDa protein. The NaOH-extracted antigen of *E. rhusiopathiae* was electrophoresed, transferred onto an Immobilon membrane, cut into strips and then reacted with MAbs of Kn-L (Lane 1), Kn-E (Lane 2), Kn-D (Lane 3), Kn-F (Lane 4), Kn-Q (Lane 5) and Kn-R (Lane 6). Molecular mass markers are shown on the left (Lane M).

Table 1. Passive immunizing efficacy of MABs

MAB	Immunoglobulin subclass	Survival rate ^{a)}
Kn-L	IgG ₁	10/10
Kn-E	IgG ₁	10/10
Kn-D	IgG ₁	9/10
Kn-F	IgG ₁	8/10
Kn-Q	IgG _{2b}	0/10
Kn-R	IgG _{2b}	0/10
Control ^{b)}	•	0/10

a) Number of mice surviving/inoculated. b) The control mice were inoculated with normal mouse serum.

group B showed a similar pattern to MAb Kn-L (data not shown).

Protective effect of bacterin (inactivated whole bacterial cells) and ELISA antibody response against 67 kDa protective antigen: The bacterins induced protection of varying degrees against infection with strain Fujisawa and different levels of ELISA antibody production against the 67 kDa protective antigen in mice. The bacterins prepared from strains Shizuoka-63, Saitama-1, CN3461 and SE-9 gave 80 to 100% protection and antibodies of more than 64.0. The bacterins prepared from strains 82-875, Shizuoka-10, 82-482, 82-930, and T199 did not confer protection and antibodies of less than 15.0 (Table 2).

Fig. 2. Immunoblotting analysis of the solubilized antigens of *E. rhusiopathiae*. The antigens were prepared from eleven wild strains and four reference strains. The membranes reacted with MAb Kn-L. Molecular mass markers are shown in Lane M.

Table 2. Protective effect of bacterin against infection with strain Fujisawa and serum antibody titers against 67 kDa protective antigen

Strain	Reactivity to MAb Kn-L in immunoblotting analysis	Experiment 1 ^{a)}	Experiment 2	
		Survival rate ^{b)}	Survival rate	Antibody titer
Shizuoka-63	strong	5/5	5/5	70.8
82-510	strong	2/5	4/5	64.6
Saitama-1	strong	5/5	4/5	66.6
CN3461 ^{c)}	strong	4/5	4/5	64.0
SE-9 ^{d)}	strong	5/5	5/5	65.5
82-598	moderate	1/5	1/5	16.9
82-875	moderate	0/5	0/5	14.4
Tama-96	moderate	2/5	2/5	20.8
Shizuoka-10	moderate	0/5	0/5	14.6
CN3342 ^{e)}	moderate	3/5	1/5	13.9
82-482	weak	0/5	0/5	10.5
82-930	weak	0/5	0/5	11.5
T199	weak	0/5	0/5	13.9
44	weak	3/5	1/5	18.0
AN-4 ^{f)}	weak	1/5	1/5	13.0
Control ^{g)}	•	0/5	0/5	9.8

a) Experiment 1 was carried out only as a protection test using bacterins. b) Number of mice surviving / inoculated. c)~f) Four bacterial strains were used as references which were obtained via the National Veterinary Assay Laboratory from the National Animal Disease Center, Ames, Iowa. g) The control group was not immunized.

DISCUSSION

In countries where swine erysipelas is enzootic, vaccination is routinely practiced. Although a live-attenuated vaccine has been widely used in Japan [8, 12, 17], most other countries use an inactivated vaccine to prevent the disease [21]. This inactivated vaccine, called adsorbate bacterin, is prepared by the method of Traub [14, 16, 20]. Protective immunity has been induced by these vaccines. Several investigators have demonstrated that a 66–64 kDa protein antigen released from the cell surface of *E. rhusiopathiae* was protective in mice and pigs [3, 4, 18, 19]. However the nature of this antigen has not been well understood. We generated MAbs directed against 67–64 kDa protein of *E. rhusiopathiae* (serovar 2), and revealed that the 67 kDa protective antigen showed quantitative diversities among the strains.

MAbs obtained in this study recognized only 67 kDa protective antigen, which was considered to be identical to the 67–64 kDa protein mentioned in a previous study [5]. In immunoblotting analysis, one group of MAbs (Kn-Q and R) showed stronger reactivity for 67 kDa protective antigen than the other group (Kn-E, D, F and L) (Fig. 1), suggesting that MAbs Kn-Q and R might bind with a repeat domain of the molecule [9]. MAbs Kn-E, D, F and L were protective in passive immunization testing, but Kn-Q and Kn-R were not. Sato *et al.* reported that MAbs directed against both 67 kDa and its 43 kDa derivative were classified into three groups by passive immunization testing [11]. Two of them were related to protective activity but the remaining one was not. Our present results suggest that MAbs Kn-E, D, F and L might recognize one of the epitopes associated with protection as described by Sato *et al.*

The protection by bacterins against infection with strain Fujisawa tended to be related to the amount of 67 kDa protective antigen on the bacterial surface. Zarkasie *et al.* reported that bacterins prepared from different strains of *E. rhusiopathiae* (serovar 2) conferred various degrees of protection in mice in spite of constancy in the expression of 67 kDa antigens detected by immunoblotting analysis [23]. In our study the *E. rhusiopathiae* strains tested expressed different levels of the 67 kDa protective antigen by the immunoblotting analysis (Fig. 2), and the bacterins containing large amounts of the antigen tended to induce a high level of antigen-specific antibody production and to give mice better protection against infection with strain Fujisawa (Table 2). These discrepancies may be due to the polyclonal and monoclonal antibody used in the detection for the 67 kDa antigen.

In conclusion, the 67 kDa protective antigen of *E. rhusiopathiae* (serovar 2) was shown to be quantitatively different among strains of serovar 2, which may reflect the protective effect of the bacterin. We propose that a strain of *E. rhusiopathiae* expressing a large amount of 67 kDa antigen should be selected to produce a bacterin or a component vaccine.

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