Presence of 22- and 17-kDa Proteins Reacting with Sera in Mice Experimentally Infected with *Brachyspira (Serpulina) hyodysenteriae*

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ABSTRACT. The antibodies to *B. (S.)hyodysenteriae* in experimentally infected mice were detected by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). The reactions in MAT were serotype specific while those in ELISA were common to both strains. A further investigation with immunoblotting technique demonstrated that 22- and 17-kDa proteins reacted strongly with the sera. The proteins in ATCC 27164 strain strongly reacted with the serum from ATCC 31212 strain-infected mouse and vice versa. These proteins were sensitive to proteinase K. — KEY WORDS: antibody, *Brachyspira*, mouse.

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Brachyspira (Serpulina) hyodysenteriae is the causative agent of swine dysentery (SD) [3, 17]. We proposed the unification of the genera *Serpulina* [15] and *Brachyspira* [4], and the use of genus *Brachyspira* [12]. Mice have been used for the experimental infectious model of SD [5]. However, there have been no reports on the antibodies in mice infected with *B.(S) hyodysenteriae*. Boyden *et al*. [2] cloned genes that code for *B.(S.) hyodysenteriae* endoflagella antigens into *E. coli* with the purpose of identifying protective antigens for vaccine development and confirmed the protective activity of the cloned endoflagella antigen using CF1 mice. However, the antibacterial activity of hyperimmune pig serum raised against axial filaments consisting of 37-, 34-, and 32- kDa proteins could not be demonstrated [10]. On the other hand, pigs that have been infected with virulent strains of *B.(S.) hyodysenteriae* and recover from the disease have been shown to be immune to further infection [6, 13]. The antibody in pig which was reacting with 16-kDa protein in *B.(S.) hyodysenteriae* was confirmed [7, 13]. A serum from a gnotobiotic pig infected with *B.(S.) hyodysenteriae* strain P18A had antibodies to the 16-kDa antigen alone and also possessed agglutinating and growth-inhibitory activities [13]. These results demonstrated that the antibodies may contribute to an antibacterial mechanism *in vivo* which could lead to protection of the pig from swine dysentery. The 16-kDa antigen was sensitive to proteinase K and consisted of lipoprotein [19]. As a further investigation, the antigen was confirmed to be encoded on *smpA* gene and its primary structure was determined by nucleotide sequencing [18]. However, there are no reports on the 22-kDa protein which may stimulate the production of antibodies. As the first step towards developing an effective subunit vaccine, we examined the antibody response of mice infected with *B.(S.) hyodysenteriae*.

B.(S.) hyodysenteriae ATCC27164 serotype 1 and ATCC 31212 serotype 2 were used and anaerobically grown on tripticase soy agar (BBL, U.S.A.), containing 5% sheep blood at 37°C for 48 hr. For the experimental infection, eighteen 4 week old male SPF mice (Slc:ICR, Nippon SLC, Japan) were used for experimental infection with *B.(S.) hyodysenteriae*. The treatment of the mice used in this test with spectinomycin (SP) was performed essentially as described by Suenaga and Yamazaki [16] since the mice become sensitive to *B.(S.) hyodysenteriae* after the treatment. The mice were fasted for 72 hr before inoculation but distilled water containing 25 μg/m*l* of SP in the case of SP-treated mice was given *ad libitum* while only distilled water in the case of SP-nontreated mice was given. *B.(S.) hyodysenteriae* ATCC 27164 and 31212 strains were harvested and suspended in tripticase soy broth (TSB) (BBL, U.S.A.). Each suspension of ATCC 27164 and 31212 strains was adjusted at 4.05×10^7 cells/m*l* and 7.17×10^7 cells/m*l*, respectively, and 0.5 m*l* of the suspension was inoculated intragastrically into the mice. In this experiment, SP-treated mice were allocated into ATCC 27164 (n=4) and 31212 (n=5) infected groups while SP-nontreated mice were allocated into ATCC 31212 infected group (n=4) and only TSB-infected group (n=5). Reisolation of the spirochetes from the feces on days 7 and 14 after inoculation and from the cecal contents on day 21 after the inoculation was carried out using blood agar containing 400 μg/m*l* of SP [14]. All mice were bled on day 21 after the inoculation.

The microscopic agglutination test (MAT) was carried out as previously described [9]. The results are presented as the reciprocal of the maximum dilution of the serum giving a 50% microscopic agglutination of the organism. An enzyme-linked immunosorbent assay (ELISA) was carried out essentially as previously described [8]. The preparation of the antigen for ELISA was carried out as previously described [1]. After harvest, the cells were suspended in 0.01 M phosphate buffer (pH 7.2), washed three times by centrifugation at 10,000 × g for 20 min and adjusted to 0.65 at a wave length of 625 nm. The cell suspension was sonicated for 10 min (100 W, 10 kHz) by a sonicator UR-200P (TOMY Seiko Co., Ltd., Tokyo, Japan) and centrifuged at 2,000 × g for 30 min. The supernatant was used as the antigen for ELISA. Alkaline phosphatase

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Table 1. Antibody titers to the spirochetes in mice sera infected with *B.(S.) hyodysenteriae* by MAT and ELISA

Inocula	Sp-treated	Mouse No.	MAT[a] titers		ELISA[b] titers	
			ATCC27164	ATCC31212	ATCC27164	ATCC31212
ATCC27164	+	1	10[c]	<10	20	<10
		2	20	<10	40	40
		3	20	<10	40	20
		4	20	<10	40	10
ATCC31212	+	1	<10	20	<10	<10
		2	<10	10	<10	10
		3	<10	<10	<10	<10
		4	<10	20	10	20
		5	<10	<10	<10	<10
ATCC31212	–	1-4	<10	<10	<10	<10
TSB	–	1–5	<10	<10	<10	<10

a) Microscopic agglutination test.
b) Enzyme-linked immunosorbent assay.
c) The values show the reciprocal of the dilution of the serum.

conjugated goat anti-mouse IgG serum was purchased from Sigma (St. Louis, U.S.A.). The results are presented as the reciprocal of the maximum dilution of the serum giving 10% of colorimetric mean value of three positive controls.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [11]. The cells were washed with physiological saline and centrifuged at 10,000 × g for 20 min, three times. The cells were treated with 0.5% SDS at 37°C for 60 min. The supernatant was taken after centrifugation at 15,000 × g for 30 min and was used for SDS-PAGE. Immunoblotting technique was carried out essentially as previously described [10, 13] using semidry type blotter (Sartblot II-S, Sartorius AG, Germany). Treatment with proteinase K was carried out as previously described [18]. The cells were digested with 0.2% proteinase K (EC 3.4.21.14) (Sigma chemical, U.S.A.) at 50°C for 120 min, and analysed by immunoblotting technique.

B.(S.) hyodysenteriae was reisolated from all of the cecal contents in SP-treated and *B.(S.) hyodysenteriae* infected mice on day 21 after inoculation. Logarithmic values of colony forming units in the case of ATCC 27164 strain- and ATCC 31212 strain-infected mice were 7.38 ± 0.38 (mean ± standard deviation) and 7.03 ± 0.32 (mean ± SD), respectively. Antibodies to the spirochetes were detected in all of ATCC 27164 strain-infected mice and in three out of five ATCC 31212 strain-infected mice by MAT (Table 1). The reactions in MAT were serotype specific while in ELISA, the antibodies produced by the infection with one of the strains reacted with both antigens.

In the immunoblotting technique, some proteins in the strains reacted with sera from the experimentally infected mice (Fig. 1). Especially, 22- and 17-kDa proteins in *B.(S.) hyodysenteriae* ATCC 27164 reacted strongly with both sera from ATCC 27164 and 31212 strains-infected mice. The 22- and 17-kDa proteins were sensitive to proteinase K (data not shown).

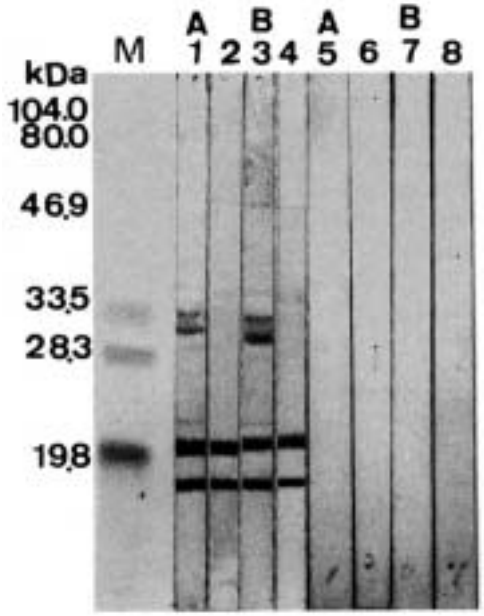

Fig. 1. Immunoblot analysis of the bacterial extracts from *B.(S.) hyodysenteriae* probing with mouse sera. Lanes: A, ATCC 27164 strain; B, ATCC 31212 strain. Lanes: 1 and 3, SP-treated and ATCC 27164 strain-infected mice sera; 2 and 4, SP-treated and ATCC 31212 strain-infected mice sera; 5 and 6, TSB-inoculated mice sera; 7 and 8, SP-nontreated and ATCC 31212 strain-infected mice sera. M, Molecular size makers.

Our results demonstrated that the antibodies to the spirochetes in experimentally infected mice were detected by MAT, ELISA and immunoblotting technique. Especially, the immunoblotting technique demonstrated that 22- and 17-kDa proteins in *B.(S.) hyodysenteriae* reacted strongly with the sera from the infected mice. The proteins in *B.(S.)*

hyodysenteriae ATCC 27164 reacted with the sera from ATCC 31212 strain-infected mice and vice versa, while the sera did not react with the proteins in *B.(S.) innocens* (data not shown). Therefore, these proteins could be common antigens to both strains which are different serotypes. The 17-kDa antigen could be similar to the 16-kDa antigen shown by Sellwood *et al*. [13]. The 16-kDa antigen was present on the surface of the spirochetes and was common to all eleven strains of *B.(S.) hyodysenteriae* but not present in the two nonpathogens [13]. The antibodies to the 16-kDa antigen also had *in vitro* antibacterial activity and the antigens also reacted with sera of a pig affected with SD [13]. Thomas *et al*. [19] speculated that the 16-kDa antigen may have some value as a component in the control of SD. On the other hand, there are no reports on the 22-kDa protein in *B.(S.) hyodysenteriae* but the protein strongly reacted with sera from the infected mice, the same as 17-kDa protein. The 22-kDa protein may also play a role in stimulation of the protective immune response.

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