Relationship Between Anticapsular Antibody and Protective Activity of a Capsular Antigen of *Haemophilus paragallinarum*

Akira SAWATA, Katsumi KUME¹⁾, and Toyotsugu NAKAI

Research Center for Veterinary Science, The Kitasato Institute, Kashiwa, Chiba 277, and ¹⁾The Kitasato Institute, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan

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ABSTRACT. Relationship between anticapsular antibody and protective activity of a capsular antigen of *Haemophilus paragallinarum* serotype HA-1 strain 221 was investigated. A capsular antigen was extracted from encapsulated organisms of the hi variant by various procedures. Indirect hemagglutination (IHA) test was used for detecting anticapsular antibodies. The IHA antibodies against a formalin-saline-extracted and phenol-treated antigen prepared with hi variant (hi-FSEx-P antigen) were specifically found in hi-NT antisera prepared from nontreated (NT) antigens of hi variant, and IHA activity of the antibodies found in hi-NT antiserum was specifically inhibited by the hi-FSEx-P antigen. The capsular antigen was found to be heat-stable and hyaluronidase-resistant. Chicks injected intravenously with hi-FSEx-P antigen produced anticapsular antibodies, and protection against the challenge exposure with 221-hi organisms was not observed in these chicks despite the presence of IHA antibodies. The results showed that protective activity of *H. paragallinarum* dose not reside in a capsular antigen, and that anticapsular antibody formation significantly depends on a route of injection and on ages of chicks.—**Key words:** anticapsular antibody, capsular antigen, *Haemophilus paragallinarum*.

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INTRODUCTION

Haemophilus influenzae possess serotype-specific capsular antigens. Anticapsular antibodies were shown to confer immunity to invasive disease caused by *H. influenzae* [24]. Little is known about roles of capsular antigen and anticapsular antibodies in the infections coryza in chicks caused by *Haemophilus paragallinarum*.

Various studies showed that encapsulated organisms formed iridescent colonies and were virulent in chickens, whereas nonencapsulated ones formed noniridescent colonies and were avirulent [7, 13, 19, 21]. Therefore, virulence of *H. paragallinarum* seems to be related to capsular substance or substance specifically produced from encapsulated organisms [19].

Serotype-specific agglutinogens [6, 18, 21]

or hemagglutinins [11, 23], but not capsule antigen [20] of *H. paragallinarum* have been shown to be related to their immunogenicity [9]. Encapsulated organisms of *H. paragallinarum* serotype HA-1 [11] (serotype 1 [21]) lacked agglutinability, since the capsule inhibited agglutination with homologous antisera and hemagglutination with freshly collected and glutaraldehyde-fixed chicken erythrocytes (RBC) [19, 20]. The inhibition was easily eliminated by treatment with hyaluronidase. Both encapsulated and nonencapsulated organisms had variant-specific (serotype-specific [11]), heat-labile, trypsin-sensitive, hyaluronidase-resistant agglutigen “L” and hemagglutinin “HA-L” [19]. Protective immunity was induced in chicks by inactivated whole cell vaccines prepared from encapsulated or nonencapsulated organisms possessing both antigens L and HA-L, where-

as protection was not induced by vaccines prepared from slightly encapsulated or nonencapsulated organisms lacking the two antigens, L and HA-L [9]. These results indicate that both antigens L and HA-L of *H. paragallinarum* are not related to the capsules. Recently, Sawata *et al.* [20] suggested that location of HA-L hemagglutinin is at outer membrane of the bacterial cells. In the present study, protective activity of a capsular antigen was investigated in relation to anticapsular antibodies by the use of altered cells and extracted antigens prepared from encapsulated organisms of *H. paragallinarum*.

MATERIALS AND METHODS

Bacterial strains: Five variants designated hi, ni-A, ni-B, R-A, and R-B were used. The variants were dissociated from strain 221 [8] of *H. paragallinarum* belonging to a serotype HA-1 [11] (serotype 1 [21]), and their properties were reported in a previous paper [9, 19].

Media: Sawata's (S) agar medium [12] was used as a solid medium. A liquid medium (S broth medium) identical to S agar medium except that agar (Difco) was omitted, and used for cultures to prepare agglutinin, hemagglutinin, and immunogen.

Electron microscopy: Microscopic observations for capsules were made on negatively-stained preparations of bacterial cells. The cells grown on S agar medium at 37°C for 5 hr were suspended in 0.5 ml of 0.02 M saline solution at pH 7.0, and the cell suspension (about 10^{10} viable cells/ml) was used. Preparation of microscopic specimens was followed by the method described by Swaney and Breese [25]. Electron microscopic observation was made with JEM-100 electron microscope (Japan Electric Co.) operating at 80 Kv.

Antigens: The variants were grown in S broth medium at 37°C for 12 hr, the bacterial cells were harvested by centrifugation at $10,000\times g$ for 30 min at 4°C, and the following antigens were prepared according to the

procedures described previously [19]: non-treated (NT), hyaluronidase (Sigme, type VI-S, from bovine testis, product No. H-3631)-treated (HU), trypsin (P-L Biochemical)-treated (T), 2 M saline and trypsin-treated (SAT), heated at 100°C for 1 hr at pH 7.0 (HE 100), and heated at 121°C for 2 hr at pH 7.0 (HE 121) antigens. All the antigens were adjusted to the concentration of a 1×10^{11} cells/ml by spectrophotometry (650 nm , 1×10^{10} cells/ml=optical density of 0.386) with model 6/20 of spectrophotometer (Coleman Instruments), and used for rapid plate agglutination (RPA) and hemagglutination-inhibition (HI) tests after addition of 0.01% thimerosal.

Extracted antigens: Various kinds of extracted antigens with variants hi, ni-A, or ni-B were prepared from the cultures grown in S broth medium at 37°C for 12 hr. Cells were collected by centrifuging at $10,000\times g$ at 4°C for 30 min, washed once in 0.01 M phosphate-buffered saline solution (PBS, pH 7.0), and suspended in 0.15 M saline solution containing 0.5% formalin, or in PBS, at a concentration of 5×10^{11} cells/ml. Three kinds of antigens were prepared as follows: i) Formalin-saline-extracted antigen, designated "FSEx", was prepared from the cell suspension in saline solution containing formalin. The suspension was agitated with magnetic stirrer at room temperature for 72 hr. After centrifugation at $20,000\times g$ at 4°C for 45 min, supernatant was collected and dialized against PBS at 4°C for at least 24 hr. The antigen was stored at -80°C. ii) Formalin-saline-extracted and phenol-treated antigen, designated "FSEx-P", was prepared from the FSEx antigen treated with phenol as follows; an equal volume of 90% phenol was mixed with FSEx antigen, the mixture was agitated in an ice bath for 20 min, and then centrifuged at $5,000\times g$ for 20 min. The aqueous phase was removed from the tube, and the extraction was repeated until interface between the aqueous and the phenol layer

completely disappeared. The resulting fraction was dialyzed against distilled water at 4°C for at least 48 hr, and was stored in a lyophilized state. iii) Sonic-extracted crude antigen, designated "S", was prepared from the cell suspension in PBS. Sonication was repeated twice with model 150 of sonicator (Otake Sonicator Co.) at 10 kilocycle each for 10 min. After centrifugation at $20,000 \times g$ at 4°C for 45 min, the resulting supernatant was collected, and was stored at -80°C.

Antisera: Rabbits were injected with various antigens in the amount corresponding to approximately 10^9 cells. The subcutaneous injections were done twice a week followed by six intravenous administrations with 3-day intervals [22].

Agglutination test: The results of the RPA test were read according to the criteria described previously [22].

Erythrocytes (RBC): Glutaraldehyde (GA, Eastman Kodak)-fixed RBC from 60-day-old line S chicks (Nisseiken Co.), were used for HI test [23]. The GA-fixed RBC were prepared by the method of Bing *et al.* [2]. Freshly collected RBC of 60-day-old line S chicks were used for indirect-hemagglutination (IHA) test.

HI test: HI test was performed by the method described previously [23].

IHA and IHA inhibition tests: The IHA test described by Crumpton *et al.* [5] was modified as follows: FSEx-P antigens from variants or S antigen from hi variant (hi-S antigen) treated by heating at 121°C for 2 hr (hi-S-HE 121 antigen) were used for coating fresh chicken RBC. Equal volumes of the 20% RBC suspension and the antigen diluted 1:80 of original volume with PBS were mixed and incubated at 37°C for 90 min. These sensitized RBC were washed three times with PBS and suspended in 0.5% in PBS. Serum samples were absorbed with 5% fresh chicken RBC suspension in 1:5. Two-fold serial dilutions (0.4 ml/tube) starting with 1:10 dilution were made and equal amounts of sensitized

RBC suspension were added. The results were read after 2 hr as the highest serum-dilution showing complete IHA.

The IHA inhibition test was performed as follows: the hi-NT antiserum with IHA titer 1:1, 280 against hi-FSEx-P antigen was used as the test antiserum. Two-fold serial dilutions (0.2 ml/tube) starting with 1:2 dilution were made of each extracted antigen with PBS. To each tube, equal amounts of 221-hi-NT antiserum with 4 IHA units/0.2 ml were added and left at room temperature for 20 min. Then, 0.4 ml of the 0.5% hi-FSEx-P antigen sensitized RBC suspension was added. After standing at 2 hr at room temperature, the results were read at the maximum antigen-dilution that inhibited IHA completely.

Gel diffusion test: Gel diffusion tests were done with a standard four-well Ouchterlony pattern in 1% agarose. The precipitin lines appeared were stained with Amido Black 10 B or Pass reagent.

IHA inhibition test of extracted antigens: Extracted antigens from 221-hi cells by treatment with hyaluronidase, by heating at 100°C for 1 hr, or at 121°C for 2 hr were investigated for their inhibitory effects of IHA by the above mentioned procedure to detect capsular antigen involved in these extracted antigens.

Biologic properties of a capsular antigen: Extracted antigens involving a capsular antigen were treated by heating at 100°C for 1 hr or at 121°C for 2 hr. Treatment with hyaluronidase was done as follows: 100 units/ml of hyaluronidase was placed in hi-S or hi-FSEx-P antigens, and the mixtures were incubated at 37°C for 2 hr. Then, treated antigens were heated at 100°C for 30 min to abolish the effect of hyaluronidase to RBC. These treated antigens were investigated for their IHA inhibition titers.

Immunization of chicks: Seven or 13-week-old line S specific-pathogen-free chicks were used for the immunogenic tests. The concentration of each of the antigen FSEx,

Fig. 1. Negatively-stained encapsulated cell from hi variant of serotype HA-1 strain 221 of *H. paragallinarum*. Magnification, $\times 58,000$.

Fig. 2. Negatively-stained nonencapsulated cell from ni-A variant of serotype HA-1 strain 221 of *H. paragallinarum*. Magnification, $\times 58,000$.

FSEx-P, or S prepared with 221-hi variant was adjusted to correspond to a 1:16 IHA inhibition titer against 221-hi-NT antiserum (IHA titer against hi-FSEx-P antigen is 1:1,280), and used for intravenous (iv) injection in chicks. For intramuscular (im) injection in chicks, finally 900 μg of $\text{Al}(\text{OH})_3/\text{ml}$ was added in each iv inoculum.

Injection and challenge exposure: Immunologic tests were performed as follows: Group A): Each immunogen (1 ml/chick) was injected iv into 13-week-old chicks followed by three times iv injections at 1 week intervals. At 18 weeks of age, chicks were interanasally challenge-exposed with 221-hi variant [6.7×10^8 colony-forming units (CFU)/0.2 ml/chick]. The chicks were euthanatized at 19 weeks of age. Group B): Each immunogen (1 ml/chick) was injected iv into 7-week-old chicks followed by three times weekly iv injections. At 12 weeks of age, chicks were challenge-exposed with 221-hi variant (6.7×10^8 CFU/0.2 ml/chick). The chicks were euthanatized at 13 weeks of age. Group C): Each immunogen (1 ml/chick) was injected in into 7-week old chicks followed by two times im injections at 3-weeks intervals. At 16 weeks of age, chicks

were challenge-exposed with 221-hi variant (7.9×10^8 CFU/0.2 ml/chick). The chicks were euthanatized at 17 weeks of age. The protective immunity was determined by the procedures reported previously [13].

RESULTS

Electron microscopic observation: Negatively-stained cells from variants hi, ni-A, ni-B, R-A, and R-B, were examined under electron microscope. Capsules were apparently seen with hi cells (Fig. 1), but not with ni-A, ni-B, R-A, or R-B cells (Fig. 2). Pili and flagella were not observed.

Serologic properties of capsular antigen: To estimate various antibody responses of each variant, antisera prepared from NT antigens of the variants were examined by IHA, HI, and RPA tests (Table 1). Antisera prepared from hi-NT antigen showed IHA titers from 1:640 to 1:2,560 against hi-FSEx-P antigen, whereas those prepared from ni-A-NT, ni-B-NT, R-A-NT, or R-B-NT antigens did $<1:20$. Thus, IHA antibodies against hi-FSEx-P antigen were exclusively found in the hi-NT antiserum. Antisera prepared from hi-NT, ni-A-NT, and R-A-NT antigens which possess variant-specific antigens L and HA-

Table 1. Serologic properties of antisera prepared from nontreated (NT) antigens of variants dissociated from serotype HA-1 strain 221 of *H. paragallinarum*

Antiserum Against	IHA ^{a)} titers with hi-FSEx-P ^{b)} antigen	HI ^{c)} titers with hi-HU ^{d)} antigen	RPA ^{e)} titers with hi-HU ^{d)} antigen
hi-NT	1:640–1:2,560	1:160–1:640	1:80–1:640
ni-A-NT	<1:20	1:640–1:2,560	1:160–1:1,280
ni-B-NT	<1:20	<1:20	<1:20
R-A-NT	<1:20	1:160–1:320	1:40–1:320
R-B-NT	<1:20	<1:20	<1:20

a) Indirect hemagglutination.

b) Formalin-saline-extracted and phenol-treated.

c) Hemagglutination-inhibition.

d) Hyaluronidase-treated.

e) Rapid plate agglutination.

Results were obtained by using five lots of antisera in each variant.

Table 2. Indirect hemagglutination inhibition activity of extracted antigens prepared with variants hi, ni-A, and ni-B dissociated from serotype HA-1 strain 221 of *H. paragallinarum* against 221-hi-NT antiserum

Extracted antigen	IHA inhibition titers against 221-hi-NT antiserum ^{a)}
Negative control (PBS)	<1:2
hi-S ^{b)}	1:512–1:1,024
hi-FSEx ^{c)}	1:256–1:512
hi-FSEx-P	1:128–1:256
ni-A-S	<1:2
ni-A-FSEx	<1:2
ni-A-FSEx-P	<1:2
ni-B-S	<1:2
ni-B-FSEx	<1:2
ni-B-FSEx-P	<1:2

a) IHA titer of 221-hi-NT antiserum was 1:1, 280 against hi-FSEx-P antigen.

b) Sonic-extracted.

c) Formalin-saline-extracted.

Results were obtained by using five lots of extracted antigens in each antigen.

L, showed HI activities with titers from 1:160 to 1:2,560 and agglutinins (L agglutinin) from 1:40 to 1:1,280 against hi-HU antigen, but those prepared from ni-B-NT and R-B-NT antigens, lacking antigens L and HA-L, showed titers of <1:20 for both HI and agglutination activities with hi-HU antigen. These results indicated that only hi variant can produce antibodies against hi-FSEx-P

antigen.

If extracted antigens from hi variant are responsible for IHA activity, absorption of antisera with the antigen showed eliminate the activity. To prove this point, 221-hi-NT antiserum having high IHA activity against hi-FSEx-P antigen was examined by IHA inhibition test (Table 2). The results showed that IHA activity of 221-hi-NT antiserum was

Fig. 3. Gel diffusion analysis of extracted antigens prepared from encapsulated organisms of hi variant of *H. paragallinarum* serotype HA-1. Well 1 contained rabbit 221-hi-NT antiserum (IHA titer is 1:1,280); wells 2, 3, and 4 contained antigens hi-S, hi-FSEx, and hi-FSEx-P, respectively. Stained by amido black 10 B.

Table 3. Extraction of a capsular antigen from 221-hi cells of *H. paragallinarum* by various treatments

Supernatants collected from variously treated antigens	IHA inhibition titers against 221-hi-NT antiserum ^{a)}
Hyaluronidase-treated	1:128–1:256
Heated at 100°C for 1 hr	1:16–1:32
Heated at 121°C for 2 hr	<1:2
Negative control (PBS)	<1:2
Positive control (hi-FSEx-P antigen)	1:128–1:256

a) IHA titer of 221-hi-NT antiserum was 1:1, 280 against hi-FSEx-P antigen.

Results were obtained by using five lots of supernatants in each antigen.

specifically inhibited by the extracted antigens S, FSEx, and FSEx-P with 221-hi variant.

The gel diffusion analysis showed that hi-S and hi-FSEx antigens contained at least three antigens and that only one of the three antigens was retained in hi-FSEx-P fraction (Fig. 3). The common antigen was stained positively with Pass reagent, indicating a high carbohydrate content. The results of IHA, IHA inhibition, and gel diffusion tests showed that extracted antigens from hi variant contain various amounts of capsular antigen, and that anticapsular antibodies can be detected by the IHA test.

Characteristics of a capsular antigen: A capsular antigen was significantly extracted from 221-hi cells by treatment with hyaluronidase, or extracted by heating at 100°C for 1 hr with less yield, but not extracted by heating at 121°C for 2 hr (Table 3). Extracted antigens involving a capsular antigen was treated with hyaluronidase or heating, and IHA inhibition titers were determined (Table 4). The capsular antigen was shown to be heat-stable and hyaluronidase-resistant.

Anticapsular antibody-producing antigenicity was slightly reduced by heating at 100°C for 1 hr, and the antigenicity was significantly reduced by treatment with hyaluronidase

Table 4. Biologic properties of extracted antigens involving a capsular antigen prepared with hi variant dissociated from serotype HA-1 strain 221 of *H. paragallinarum*

Extracted antigen involving a capsular antigen	Treatment	IHA inhibition titers against 221-hi-NT antiserum ^{a)}
hi-S	None	1:512–1:1,024
	Hyaluronidase	1:256–1:512
	Heated at 100°C for 1 hr	1:128–1:256
	Heated at 121°C for 2 hr	1:128–1:256
hi-FSEx-P	None	1:128–1:256
	Hyaluronidase	1:64–1:128
	Heated at 100°C for 1 hr	1:32–1:64
	Heated at 121°C for 2 hr	1:16–1:32
Negative control (PBS)		<1:2

a) IHA titer of 221-hi-NT antiserum was 1:1, 280 against hi-FSEx-P antigen.

Results were obtained by using five lots of extracted antigens in each treatment.

Table 5. Antigenicity of variously treated antigens prepared with 221-hi variant of *H. paragallinarum*

Antiserum against	IHA titers with hi-FSEx-P antigen	IHA titers with hi-S-HE 121 antigen	HI titers with hi-HU antigen	RPA titers with hi-HU antigen
hi-NT	1:640–1:2,560	1:320–1:1,280	1:160–1:640	1:80–1:640
hi-HU	<1:20–1:40	<1:20	1:320–1:2,560	1:80–1:1,280
hi-T ^{a)}	<1:20	<1:20	<1:20	<1:20
hi-SAT ^{b)}	<1:20	<1:20	<1:20	<1:20
hi-HE 100 ^{c)}	1:160–1:320	1:160–1:320	<1:20	<1:20
hi-HE 121 ^{d)}	<1:20	<1:20	<1:20	<1:20

a) Trypsin-treated.

b) 2 M saline and trypsin-treated.

c) Heated at 100°C for 1 hr.

d) Heated at 121°C for 2 hr.

Results were obtained by using five lots of antisera in each antigen.

(Table 5). Treatment with trypsin or heating at 121°C for 2 hr (HE 121 antigen) abolished the antigenicity. The hi-S-HE 121 antigen, however, could be used for the IHA test. The results showed that the antigen retained ability to combine with anticapsular antibodies, but it lost the anticapsular antibody-producing antigenicity by the heating.

Protective activity of a capsular antigen: Chicks injected iv or im with hi-S, hi-FSEx, or hi-FSEx-P antigen (vaccine) involving a

capsular antigen in a varying extents were challenge-exposed with 221-hi variant (Table 6). Chicks injected iv with hi-FSEx or hi-FSEx-P antigen produced anticapsular antibodies defined by the IHA test, whereas the antibodies could not be detected in all the im injected chicks with hi-S, hi-FSEx, and hi-FSEx-P antigens. Protection against the challenge exposure with 221-hi variant was observed in chicks having HI antibodies and L agglutinins against hi-HU antigen, which

Table 6. Protective activity of chicks injected iv or im with extracted antigens involving a capsular antigen FSEx, FSEx-P, or S prepared with 221-hi variant of *H. paragallinarum* against intranasal challenge exposure with 221-hi organisms

Immunogen (No. of chicks challenge-exposed ^a)	Serum antibody when challenge-exposed					
	Geometric mean of IHA titers with 221-hi-FSEx-P antigen	Geometric mean of HI titers with 221-hi-HU antigen	No. of RPA positive (>5) chicks with 221-hi-HU antigen	No. of chicks with clinical signs of coryza	No. of chicks from which challenge exposure strain was recovered	No. of chicks infected ^b)
Group A						
221-hi-FSEx (6)	1,440 ^{a,a})	20.4	2	0	1	1
221-hi-FSEx-P (6)	1,447 ^{a,a})	<2.5	0	6	6	6
Control (3)	<5	<2.5	0	3	3	3
Group B						
221-hi-FSEx (6)	160 ^{b,b})	7.1	1	1	1	1
221-hi-FSEx-P (6)	80 ^{b,b})	<2.5	0	6	6	6
Control (3)	<5	<2.5	0	3	3	3
Group C						
221-hi-S (10)	<5	73.4	9	1	1	1
221-hi-FSEx (10)	<5	5.5	2	4	8	8
221-hi-FSEx-P (10)	<5	<2.5	0	10	10	10
Control (5)	<5	<2.5	0	5	5	5

a) Challenge exposure doses [(A)= 6.7×10^8 CFU/0.2ml/chick; (B)= 6.7×10^8 ; and (C)= 7.9×10^8].

b) No. of chicks with clinical signs of coryza and/or from which challenge exposure strain was recovered.

For each column, means with lettered superscripts are different (double letters, $P < 0.01$).

Group A: Inoculated iv at 13, 14, 15, and 16 weeks of age, challenge-exposed at 18 weeks of age.

Group B: Inoculated iv at 7, 8, 9, and 10 weeks of age, challenge-exposed at 13 weeks of age.

Group C: Inoculated im at 7, 10, and 13 weeks of age, challenge-exposed at 16 weeks of age.

were injected iv with hi-FSEx antigen and in chicks injected im with hi-S and hi-FSEx antigens. Highly significant differences ($P < 0.01$) were observed in protection rate between hi-FSEx and hi-FSEx-P antigens of both iv injection groups A and B as well as between hi-S and hi-FSEx-P antigens of im injection group C.

Highly significant differences ($P < 0.01$) were also observed in anticapsular antibody titers of hi-FSEx and hi-FSEx-P antigens between iv injection groups A and B. The results showed that protective activity of *H. paragallinarum* serotype HA-1 dose not reside in a capsular antigen, and that anticapsular antibody formation significantly depends on route of injection and on ages of chicks. A capsular antigen and anticapsular antibody-producing antigenicity were found only in encapsulated organisms of hi variant, but, protective immunity was found in variants hi, ni-A, and R-A with antigens L and HA-L. There is no correlation between anticapsular antibody and protective activity.

DISCUSSION

The previous report concerning the phenotypic forms of *H. paragallinarum* serotype HA-1 [11] (serotype 1 [21]) showed that location of variant-specific antigens L and HA-L may not reside in capsules [9]. In addition, protective activity of *H. paragallinarum* serotype HA-1 seems not to be reside in capsular antigen, because significant difference in protection rate was not observed between chicken groups immunized with non-treated (NT) and hyaluronidase-treated (HU) vaccines prepared from encapsulated organisms of hi variant [9]. The treatment with hyaluronidase apparently removed capsules from the cells of hi variant [9], when the treated cells (hi-HU antigen) were determined by Möller's capsule-staining method [16]. These results indicate that both antigens L and HA-L are not of capsular origin. In the present investigation, we attempted to detect

anticapsular antibodies by IHA test, and to investigate the relationship between anticapsular antibody and protective activity of a capsular antigen of *H. paragallinarum* serotype HA-1.

Evidence is apparent that anticapsular antibodies can be detected by the IHA test. The IHA activity against formalin-saline-extracted and phenol-treated antigens prepared from encapsulated organisms of hi variant (hi-FSEx-P antigen) was found in antisera prepared from NT antigens of hi variant (hi-NT antiserum), but not in the antisera prepared against NT antigens of nonencapsulated variants ni-A, ni-B, R-A, and R-B (Table 1). The FSEx-P antigen prepared with hi variant (hi-FSEx-P antigen) specifically inhibited this IHA activity, whereas FSEx-P antigens prepared with variants ni-A or ni-B lacked this property (Table 2). On the other hand, hi-FSEx-P antigen prepared with hi-variant could not produce HI antibodies against HA-L hemagglutinin and L agglutinins, when the antigen was injected im or iv into both chicks or rabbits. Furthermore, hi-FSEx-P antigen contained one antigen by gel diffusion test (Fig. 3). These results indicate that hi-FSEx-P antigen contains a capsular antigen, but that it lacks variant-specific antigens L and HA-L.

Previously, Sawata and Kume [19] reported that both antigen L for agglutination and HA-L hemagglutinin, which presented at outer membranes of bacterial cells [20], were completely inactivated by heating at 65°C for 30 min or by treatment with trypsin, but not by treatment with hyaluronidase. Our previous results showed that the antigens L and HA-L seemed to be protein in nature [19, 22, 23]. A capsular antigen of *H. paragallinarum* serotype HA-1, however, was found to be stable by heating at 100°C for 1 hr or by treatment with hyaluronidase (Table 3). Therefore, by using the difference in heat-sensitivities, a capsular antigen can be easily distinguished from a kind of somatic antigens L and HA-L. A capsular antigen of *H.*

paragallinarum seems to be a polysaccharide in nature, when it was stained with Pass reagent.

Hinz [7] previously reported that capsular antigen presented both in culture filtrates and in bacterial washings prepared from encapsulated organisms of *H. paragallinarum* serotypes A and B [18], whereas capsular antigen was absent in those prepared from nonencapsulated organisms. The author [7] used thus nontreated samples as capsular antigen for coating RBC. A capsular antigen of *H. paragallinarum* serotype HA-I can be extracted from encapsulated organisms by sonication, treatment with formalin and saline (Table 2), treatment with hyaluronidase, or heating at 100°C for 1 hr (Table 3). However, these extracted antigens prepared from the cells of encapsulated hi variant contained not only a capsular antigen but also a kind of somatic antigens L and HA-L in a varying extents. Treatment of these extracted antigens with phenol or by heating at 100°C for 1 hr are the effective methods to abolish a protein-like antigens L and HA-L without significant loss in a capsular antigen.

However, when treated by heating at 121°C for 2 hr, the hi-S or hi-FSEx-P antigens still showed IHA inhibition activity against anticapsular antibodies presented in 221-hi-NT antiserum (Table 4). The hi cells treated by heating at 121°C for 2 hr could not produce anticapsular antibodies in rabbits (Table 5). These results indicate that a capsular antigen of *H. paragallinarum* serotype HA-I treated the procedure retained ability to combine with anticapsular antibodies, but it lost completely the anticapsular antibody-producing antigenicity.

In the report concerning the protective activity of *H. paragallinarum* serotype HA-I [9, 10, 12–15, 23], Kume *et al.* reported that protective immunity resided in both antigens L and HA-L. The results of present study confirmed the observations in that protection was observed only in chicks with HI antibodi-

es against HA-L hemagglutinin and L agglutinins. Protective immunity was not correlated with levels of anticapsular antibodies. However, the fact does not eliminate the possibility that the capsular antigen is involved in the pathogenesis of infectious coryza [19].

It is apparent that the route of injection and ages of host have significant influence on the production of anticapsular antibodies. The reason why anticapsular antibodies can not be produced in the chicks injected im three times remained uncertain. However, this situation strongly supports our present observation that a capsular antigen of *H. paragallinarum* serotype HA-I completely lacks protective activity, because the vaccines prepared from *H. paragallinarum* serotype HA-I produced significant levels of both HI antibodies against HA-L hemagglutinin and L agglutinins even two to three weeks after primary im injection in chicks, and most of these chicks were significantly protected against the challenge exposure with homologous challenge exposure strains [9, 10, 12–15, 23].

H. influenzae possess serotype-specific capsular antigens, and anticapsular antibodies can be detected by serologic procedures [1, 3, 4, 17, 26]. Hinz [7] reported the presence of serotype-specific capsular antigens of *H. paragallinarum* serotypes A and B by means of the IHA test. Furthermore, the strains classified into serotypes A and B by the IHA test significantly corresponded to those classified into the serotypes A and B by the agglutination test [6]. Sawata and Kume [19] reported that encapsulated organisms of serotype HA-I lacked agglutinating activity with homologous antisera and hemagglutinating activity with chicken RBC, by the presence of capsules, but the treatment with hyaluronidase restored these activities. The treatment with hyaluronidase could extract a capsular antigen significantly from encapsulated organisms of *H. paragallinarum* serotype HA-I (Table 3). In the present investigation, it is clearly shown that biologic, serologic, and

immunologic properties of a serotype HA-I capsular antigen quite differed from those of a kind of somatic antigens L and HA-L. It is of interest to speculate whether or not serotypes classified by Hinz [7] by capsular antigens correspond to those classified by antigens L [22] or HA-L [11]. Further biochemical analysis of these antigens including a capsular antigen is necessary.

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要 約

Haemophilus paragallinarum の抗莢膜抗体と莢膜抗原の感染防御能: 澤田 章・久米勝巳¹⁾・中井豊次 (北里研究所附属家畜衛生研究所, ¹⁾北里研究所) ——*Haemophilus paragallinarum* の変異菌を用い, 本菌莢膜抗原の性状, 抗莢膜抗体産生能, ならびに莢膜抗原の感染防御能を調べた。莢膜抗原は莢膜保有 hi 型菌から種々な方法で抽出されたが, ホルマリン加食塩で抽出後, フェノール処理した hi-FSEx-P 抗原はゲル内拡散法で莢膜抗原のみを含むことが確認された。本抗原は耐熱性, ヒアルロニダーゼ抵抗性の多糖体であった。抗莢膜抗体は間接赤血球凝集 (IHA) 試験で検出でき, 本抗体の IHA 活性は莢膜抗原で特異的に阻止されたため, 莢膜抗原の量は IHA 抑制価で測定しえた。莢膜抗原免疫鶏は感染防御能を欠き, かつ, 感染防御は抗莢膜抗体の有無とは無関係に, 特定の菌体抗原に対する抗体保有鶏で認められ, 感染防御能は莢膜に存在しないことが確認された。なお, ニワトリにおける抗莢膜抗体産生能は莢膜抗原の注射経路および注射時のひなの日齢によって異なった。