Immunologic Properties of Variants Dissociated from Serotype 1 *Haemophilus paragallinarum* Strains

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(Received 19 August 1983/Accepted 13 October 1983)

Immunogenic properties of seven variants having different morphologic, serologic, and pathogenic properties, which were dissociated from serotype 1 (serotype HA-1) *Haemophilus paragallinarum*, were investigated in chicks. Protective immunity was induced by injection with any of the four variants possessed both heat-labile, trypsin-sensitive, hyaluronidase-resistant, serotype 1-specific L1 and HA-L1 antigens. The immunized chicks had antibodies specific to serotype 1, agglutinin against L1 antigen and hemagglutination-inhibition (HI) antibodies against HA-L1 antigen. In contrast, the other three variants lacking both the antigens did not induce protection. Hyaluronidase-treatment of the hi variant consisting of highly encapsulated organisms abolished its virulence without affecting the protective activity and antibody-producing ability against antigens L1 and HA-L1. These results support our hypothesis that the antigens L1 and HA-L1, are responsible for immunogenicity.—*Key words: Haemophilus paragallinarum*, Immunogenicity, Variant.

Jpn. J. Vet. Sci. 46(1): 49–56, 1984

Numerous attempts have been made to vaccinate chickens against infectious coryza [1]. Page *et al.* [11] found cross-protection among their serotype A, B, and C strains. In contrast, Davis *et al.* [2] and Matsumoto and Yamamoto [9] reported a lack of cross-protection even within Page's serotype A [11]. Recently, Rimler *et al.* [13] found three immunotypes corresponding to Page's serotypes [11]. Little is known, however, about the antigen responsible for protective activity of the vaccines.

Sawata *et al.* [17] demonstrated the presence of two serotypes, 1 and 2, among isolates of *Haemophilus paragallinarum*. Subsequently, two immunotypes corresponding to the serotypes [17] were found [5–8]. In serotype 1 strains, two antigens, a heat-labile, trypsin-sensitive, hyaluronidase-resistant, serotype 1-specific agglutinin L1 [18] and a hemagglutinin HA-L1 [19], seemed to be closely related to the protective activity. Thus, most chicks having agglutinin against

L1 antigen (L1 agglutinin) [6, 7] and hemagglutination-inhibition (HI) antibody against HA-L1 antigen [19] are protected against challenge exposure with serotype 1 strains. Kume *et al.* [7] could not demonstrate the presence of inter-group protection as described by Rimler *et al.* [13], even using the same test strains.

More recently, we [15] clarified morphologic and serologic properties of seven variants dissociated from serotype 1 *H. paragallinarum* strains in relation to virulence in chicks, and found that both antigens L1 and HA-L1 were located under the capsule. The virulence was well correlated with the amount of capsule. The present paper deals with the immunologic properties of the variants in relation to the virulence and immunogenicity.

MATERIALS AND METHODS

Test organisms: Seven variants designated as hi, li-A, li-B, ni-A, ni-B, R-A, and R-B variants were studied. These were dissociat-

ed from serotype 1 *H. paragallinarum* [17] (serotype HA-1 [4]) strains 221, 083, W, 7411, and 7682. Morphologic and serologic properties of each variant and a procedure for obtaining the variants were described in a previous report [15].

Media: The S agar medium was used for the cultures to prepare challenge inocula [5]. The liquid medium, S broth medium, was used to prepare immunogen, agglutinin, and hemagglutinin. Compositions of these media were described previously [15].

Antigens: Cultures were grown in S broth medium at 37°C for 10 h. The cells were collected by centrifugation at $10,000 \times g$ for 20 min, and washed once in 0.02 M phosphate-buffered solution, pH 7.0, containing 0.85% NaCl (PBS). Nontreated (NT), saline-treated (SA), 2 M NaCl and trypsin (P-L Biochemical)-treated (SAT), trypsin-treated (T), hyaluronidase (Sigma, Type VI-S, from Bovine Testes, Product No. H-3631)-treated (HU) (25 to 100 U/ml), and heat-treated at 121°C for 2 h at pH 7.0 (HE 7) antigens were prepared according to the method described previously [6, 15]. Densities of all the antigens were adjusted to that of 10^{11} NT cells/ml using model 6/20 of spectrophotometer (Coleman Instruments), and 0.01% thimerosal was added.

Antisera: Antisera were prepared by injecting rabbits with NT, SAT, or HE 7 antigens prepared from 221-hi variant [15] by the procedure reported previously [3].

Hemagglutination test: The NT, HU, SA, SAT, T, and HE 7 (10^{11} cells/ml) antigens prepared from each variant were tested for hemagglutinating activity against freshly collected erythrocytes (RBC) from line S 60-day-old chicks (Nisseiken Co., Yamanashi, Japan), by the method described in a previous paper [19].

Agglutination test: The agglutination test was performed by a rapid plate agglutination (RPA) method described in an earlier report [18], using antiserum diluted 1:12.5 to

1:3,200 with PBS and NT, HU, SA, SAT, T, and HE 7 antigens.

Microscopic test: Cellular morphologies of capsule-stained (Möller's method [10]) organisms in the NT and HU antigens prepared from a hi variant [15], were observed with a model XF-31 light microscope (Nikon).

Vaccines: The NT, HU, SA, SAT, T, and HE 7 antigens were suspended in PBS containing 0.01% thimerosal, adjusted to a concentration of 5×10^9 cells/ml, and finally added 900 $\mu\text{g/ml}$ of $\text{Al}(\text{OH})_3$ as an adjuvant.

Chicks: Eggs from stock known to be free from *Haemophilus* organisms and certain bacterial and viral infections obtained from the H&N poultry farm (Pfizer Taito Co., Shizuoka, Japan) were incubated in the laboratory, and the chicks hatched were housed in a separate room. Five-week-old chicks were used for the immunogenic test.

Immunization and challenge exposure: Each vaccine (1 ml/chick) was injected intramuscularly to 5-week-old chicks. Immunologic test was performed as follows; protective immunity was determined by intranasal challenge exposure with 221-hi variant [at least 10^8 colony-forming units (CFU)/0.2 ml/chick] 3 weeks after injection. In some cases, heterologous challenge exposure with serotype 2 (serotype HA-4 [4]) strain H-18 [5] was also given intranasally (at least 10^8 CFU/0.2 ml/chick) to the injected chicks. The chicks were examined once a day for severity of nasal exudates and facial edema, and were necropsied on 7 days after challenge exposure. At necropsy, samples obtained on cotton swab from the paranasal sinus and nasal cavity of each chick were directly streaked on S agar medium. Chicks with clinically coryza signs and/or positive *Haemophilus* isolation of the challenge exposure strain were regarded as infected. Serum samples were obtained for titration of antibodies at 3 weeks after injection.

Antibody determination: 221-hi-HU antigen [15] was used for agglutination and as

hemagglutinin. To remove isohemagglutinin, sera were absorbed with 5% RBC suspension from line S 60-day-old chicks to make a final dilution of 1:5, and by standing overnight at 4°C. A RPA test was performed with thus treated supernatant (1:5) and 221-hi-HU antigen, and the result was read according to the criteria described previously [18]. The HI test was also performed using sera which had been removed isohemagglutinin, by the method described in a previous report [19].

RESULTS

1. Protective activity of variants. Protective activity of the variants was investigated (Table 1). Chicks injected with NT antigens from hi, li-A, ni-A, or R-A variants produced HI antibody against 221-hi-HU antigen (HA-L1 hemagglutinin) and agglutinin against 221-hi-HU antigen (L1 agglutinin). Forty percent to 90% of the injected chicks were protected against challenge exposure with 221-hi variant, but not against a heterologous strain, H-18 of serotype 2. Chicks injected with NT antigens from li-B, ni-B, or R-B variants did not produce either HI antibody against 221-hi-HU antigen and L1 agglutinin, and none of the injected chicks were protect-

ed against challenge exposure even with homologous strain 221.

2. Effects of hyaluronidase on agglutinability of antigens. Agglutinations were done with antigens prepared from 221, 083, W, 7411, or 7682 strains, with or without pretreatment with hyaluronidase (Table 2). Before treatment with hyaluronidase, none of the hi-NT antigens were agglutinated in the antisera, and, at the same time, they did not agglutinate fresh chicken RBC. After the enzyme treatment, both agglutination and hemagglutination reactions became positive, and the titers increased depending on the concentration of hyaluronidase (25 to 100 U/ml), however, 100 U/ml of hyaluronidase was enough to obtain the maximum titers of the reactions.

3. Effects of treatment with hyaluronidase on capsule and protective activity. In the NT antigens originating from 221-hi variant, the capsule was found in most of the cells before hyaluronidase-treatment, but not at all in the cells after the enzyme treatment (100 U/ml). Protective activity of the hyaluronidase-treated (100 U/ml) antigens (hi-HU) prepared from 221, 083, W, 7411, or 7682 strains, were compared with the nontreated antigens (hi-NT) (Table 3). Chicks injected with hi-

Table 1. Protective activity of variants dissociated from *H. paragallinarum* serotype 1 against intranasal challenge exposure with 221-hi variant

Immunogen	No. chicks immunized and challenge exposed ^{a)}	Serum antibody when challenge exposed		Protective activity			
		Geometric mean of HI titer against 221-hi-HU antigen	No. RPA test positive (≥ 5) chicks against 221-hi-HU antigen	No. with coryza signs	No. chicks with positive recovery of challenged organisms	No. chicks infected ^{b)}	Protection rate (%)
hi-NT	10	102.3	10	0	1	1	90
li-A-NT	10	36.3	10	1	2	2	80
li-B-NT	10	≤ 2.5	0	10	10	10	0
ni-A-NT	10	5.0	2	5	6	6	40
ni-B-NT	10	≤ 2.5	0	10	10	10	0
R-A-NT	10	26.8	8	3	3	3	70
R-B-NT	10	≤ 2.5	0	10	10	10	0
Control	5	≤ 2.5	0	5	5	5	—

a) Challenge exposure dose (strain 221 = 1.8×10^8 CFU/0.2 ml/chick).

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

None of the injected chicks were protected against challenge exposure with heterologous strain H-18 of serotype 2.

Table 2. Effects of hyaluronidase on agglutinogens of *H. paragallinarum* serotype 1 variants

Antigen	Hemagglutinating activity against chicken RBC with or without hyaluronidase ^{a)}		Agglutination titer with or without hyaluronidase-treatment against rabbit anti-221-hi-NT antiserum ^{b)}	
221-hi-NT ^{c)}		<2		<12.5
221-hi-HU ^{d)}	32 ^{e)}	128 ^{f)}	800 ^{e)}	1,600 ^{f)}
083-hi-NT		<2		<12.5
083-hi-HU	8	64	400	800
W-hi-NT		<2		<12.5
W-hi-HU	<2	64	<12.5	1,600
7411-hi-NT		<2		<12.5
7411-hi-HU	8	32	400	800
7682-hi-NT		<2		<12.5
7682-hi-HU	32	128	800	1,600

a) Expressed as reciprocal of the highest dilution showing hemagglutination (the titers). Initial antigen-dilution is 1:2.

b) Expressed as reciprocal of the highest dilution showing agglutination (the titers). Initial serum-dilution is 1:12.5.

c) Nontreated antigen preparation.

d) Hyaluronidase-treated antigen preparation.

e) 25 U/ml of hyaluronidase.

f) 100 U/ml of hyaluronidase.

Table 3. Effect of treatment with hyaluronidase on protective activity of *H. paragallinarum* serotype 1 variants against intranasal challenge exposure with 221-hi variant

Immunogen	No. chicks immunized and challenge exposed ^{a)}	Serum antibody when challenge exposed		Protective activity			
		Geometric mean of HI titer against 221-hi-HU antigen	No. RPA test positive (≥ 5) chicks against 221-hi-HU antigen	No. with coryza signs	No. chicks with positive recovery of challenged organisms	No. chicks infected ^{b)}	Protection rate (%)
221-hi-NT ^{c)}	10	101.0	9	0	1	1	90
221-hi-HU ^{d)}	10	98.2	8	1	1	1	90
083-hi-NT	10	29.8	9	1	2	2	80
083-hi-HU	10	36.5	8	1	2	2	80
W-hi-NT	10	76.5	10	1	1	1	90
W-hi-HU	10	100.8	10	0	0	0	100
7411-hi-NT	10	52.0	10	1	1	1	90
7411-hi-HU	10	53.5	10	0	1	1	90
7682-hi-NT	10	77.5	9	0	1	1	90
7682-hi-HU	10	87.6	9	1	2	2	80

a) Challenge exposure dose (strain 221= 5×10^9 CFU/0.2 ml/chick).

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

c) Nontreated antigen preparation.

d) Hyaluronidase-treated (100 U/ml) antigen preparation.

None of the injected chicks were protected against challenge exposure with heterologous strain H-18 of serotype 2. All the noninjected control chicks were infected.

Table 4. Protective activity of altered antigens prepared from variants dissociated from serotype 1 *H. paragallinarum* variants against intranasal challenge exposure with 221-hi variant

Immunogen	No. chicks immunized and challenge exposed ^{a)}	Serum antibody when challenge exposed		
		Geometric mean of HI titer against 221-hi-HU antigen	No. RPA test positive (≥ 5) chicks against 221-hi-HU antigen	Protection rate (%)
221-hi-NT	10	63.0	10	100
221-hi-SA	10	84.0	10	80
221-hi-T	10	≤ 2.5	0	0
221-hi-SAT	10	≤ 2.5	0	0
221-hi-HE 7	10	≤ 2.5	0	0
221-ni-A-NT	10	10.2	4	50
221-ni-A-SA	10	8.3	4	40
221-ni-A-T	10	≤ 2.5	0	0
221-ni-A-SAT	10	≤ 2.5	0	0
221-ni-A-HE 7	10	≤ 2.5	0	0
7411-R-A-NT	10	25.2	7	70
7411-R-A-SA	10	16.9	8	60
7411-R-A-T	10	≤ 2.5	0	0
7411-R-A-SAT	10	≤ 2.5	0	0
7411-R-A-HE 7	10	≤ 2.5	0	0
Control ^{b)}	10	≤ 2.5	0	—

a) Challenge exposure dose (strain 221 = 3.2×10^8 CFU/0.2 ml/chick).

b) All the noninjected control chicks were infected.

NT or hi-HU antigens produced HI antibody against 221-hi-HU antigen and L1 agglutinin, and 80% to 100% of the chicks were protected against challenge exposure with strain 221, but not against strain H-18. No significant differences in protective rate were observed among the groups injected with different antigens. Both the either protective activity and antibody-producing ability were not affected by the hyaluronidase.

4. Protective activity of altered antigens prepared from the variants. Antigens prepared from hi, ni-A, and R-A variants were variously treated and tested for protective activity (Table 4). Chicks injected with NT and SA antigens prepared from the variants produced HI antibodies against 221-hi-HU antigen and L1 agglutinins, and 40% to 100% of them were protected against challenge exposure with strain 221. Chicks injected with trypsinized (T and SAT) or heated (HE 7) antigens

from the variants did not produce either HI antibody against 221-hi-HU antigen or L1 agglutinin, and they were not protected against challenge exposure with strain 221. Protective activity and antibody-producing ability were also observed in the NT and SA antigens from li-A variant, whereas these activities were absent in the T, SAT, or HE 7 antigens from li-A variant as well as in all the NT, HU, SA, T, SAT, or HE 7 antigens from li-B, ni-B, or R-B variants (data are not shown).

5. Relationships among protective activity, virulence, and morphologic or serologic properties of the variants. A close relationship was observed between protective activity and the presence of antigens L1 and HA-L1. Regardless of the appearance of virulence in chicks and the presence of capsule, the protective activity was found in the hi, li-A, ni-A, and R-A variants, containing both L1

and HA-L1 antigens, whereas it was absent in the li-B, ni-B, or R-B variants, lacking the L1 and HA-L1 antigens. The chicks injected with variants possessing L1 and HA-L1 antigens produced L1 agglutinin, and HI antibodies against HA-L1 antigen, and were protected against challenge exposure to strains with homologous, but not against heterologous serotypes.

DISCUSSION

Previously, Sawata *et al.* [18] reported that encapsulated organisms of serotypes 1 and 2 of *H. paragallinarum* have at least three antigens: a heat-labile and trypsin-sensitive L antigen; a heat-labile and trypsin-resistant HL antigen; and a heat-stable and trypsin-resistant HS antigen. The L antigen was serologically subdivided into three factors by agglutination test: serotype 1-specific L1 antigen; serotype 2-specific L2 antigen; and common L antigen (L3 antigen) shared by serotypes 1 and 2. It has been suggested that the L antigen is a capsular antigen [18] with immunogenic activity [6]. A similar antigen has been demonstrated in *Haemophilus influenzae* [14, 20]. Sawata and Kume [15], however, demonstrated that both antigens L [18] and HA-L [19] are different in the capsular substance. More recently, Sawata *et al.* [16] suggested that serotype 1 hemagglutinins are located in outer membrane of cells. The present investigation was initiated to analyze the location of a protective antigen by studying immunogenic properties of seven variants originating from serotype 1 strains of *H. paragallinarum*.

A close relationship was observed between the appearance of protective activity and the presence of L1 and HA-L1 antigens. Protective activity was found with the organisms having both L1 and HA-L1 antigens, whereas it was absent with those lacking these antigens. The protective activity was significantly correlated with L1 and HA-L1 antigens which induced both L1 agglutinins and HI

antibodies against HA-L1 antigen. Interestingly, virulence of the organisms did not correlate with the presence of L1 and HA-L1 antigens. In contrast, the capsular antigen, which appears to be an important virulence factor(s), did not correlate to protection.

Previously, Kume *et al.* [6] reported that nonencapsulated organisms were avirulent in chicks. In addition, all the antigens prepared from nonencapsulated organisms as well as trypsinized or heated at 121°C for 2 h antigens prepared from encapsulated organisms neither hemagglutinated fresh chicken RBC (Pfizer Taito) nor induced antibodies responsible for HI and "L" agglutination reactions [6]. These antigens lacked protective activity [6]. The present investigation showed that slightly encapsulated or nonencapsulated organisms of li-B, ni-B, or R-B variants lacked both L1 and HA-L1 antigens. These organisms lacked the ability, to produce HI and agglutination antibodies. In addition, these organisms lacked protective activity and were avirulent in chicks. Trypsinized (T and SAT antigens) or heated (HE 7 antigen) antigens prepared from all the seven variants showed the same characteristics. On the other hand, L antigen and HA-L1 hemagglutinin were found both with encapsulated and nonencapsulated organisms of hi, li-A, ni-A, and R-A as well as with antigens NT and SA prepared from these variants. The results showed the necessity of careful selection of colonies among the variants for preparation of an effective immunogen for chicks.

Evidence presented in this study and elsewhere [15, 16] suggested that a virulence factor(s) in chicks in terms of gross coryza lesions was different from protective antigen. When highly encapsulated viable organisms (hi variant) were intranasally inoculated, they produced gross coryza lesions in chicks [15]. On the other hand, nonencapsulated organisms lacked the pathogenicity [15]. The degree of virulence was well correlated with the amount of capsule [15]. Treatment with

hyaluronidase of the encapsulated organisms resulted in completely disappearance of capsules, therefore, a hyaluronic acid-like substance appears to be an important component in constituting capsule. However, treatment with hyaluronidase invariably inactivated the organisms, and, therefore, relationship between hyaluronic acid and virulence can not be tested *in vivo*. In the present investigation, it was demonstrated that treatment with hyaluronidase affected neither protective activity nor surface L1 and HA-L1 antigens of the encapsulated organisms (hi variant); the treated organisms (hi-HU) induced protective immunity as well as HI and agglutinin antibodies. Another evidence indicating noninvolvement of the capsule in immunogenicity is the fact that inactivated nonencapsulated organisms, such as ni-A and R-A variants, induced protective immunity as well as antibodies against L1 and HA-L1 antigens. "Capsule" of *H. paragallinarum* may contain certain substance(s) other than a hyaluronic acid-like substance. However, these evidences clearly indicate the presence of capsular material is related to the virulence of the viable organisms *in vivo*, but not to protective activity and "L" and "HA-L" antigen systems of the inactivated organisms. At present, we have no definite proof about capsular antigen. Further investigations are necessary to clarify the capsular antigen responsible for infection with and immunity to *H. paragallinarum*.

Whether cross-type protective immunity can be induced by vaccines or antigens of *H. paragallinarum* has been studied by many investigators [2, 5-9, 11-13, 19]. Rimler *et al.* [12] observed that cross-type immunity was induced in chicks after active infection with *H. paragallinarum*. On the other hand, vaccines have been known to induce only type-specific immunity [5-9, 12, 13, 19]. Since organisms used for vaccines were divorced of capsular material in comparison to fully encapsulated organisms induced in the

infected lesions. Rimler *et al.* [12] speculated that capsular material may be responsible for cross-type immunity. In the present investigation, however, fully encapsulated organisms of hi variant failed to induce cross-type immunity, when injected into chicks in the form of vaccines. In fact, there was no difference in type-specific immunity among various antigens (vaccines) prepared from fully encapsulated (hi-NT antigen), hyaluronidase-treated encapsulated (hi-HU antigen), and nonencapsulated (ni-A-NT and R-A-NT antigens) organisms. Hence, cross-type immunity observed in actively infected chicks may be attributed to factors other than capsules of *H. paragallinarum*.

ACKNOWLEDGEMENTS. The authors thank Dr. A. Ghoda, Kitasato University, Sagamihara, Japan, and Dr. M. Matsumoto, School of Veterinary Medicine, Oregon State University, Corvallis, Oregon 97331, for reviewing the manuscript, and Mrs. N. Kaneko, The Kitasato Institute, for technical assistance.

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要 約

Haemophilus paragallinarum 血清型 1 (血清型 HA-1) 菌株から作出した変異株の免疫学的性状: 久米勝巳・澤田 章 (北里研究所附属家畜衛生研究所) ——ヘモフィルス・パラガリナラム血清型 1 (血清型 HA-1) 菌株から作出した 7 種の変異株の免疫学的性状を調べた。易熱性, トリブシン感受性, ヒアルロニダーゼ耐性の L₁ (凝集原) ならびに HA-L₁ (赤血球凝集素) と名づけた 2 種の抗原はひなにおける防御とよく相関した。すなわち, 防御は L₁ と HA-L₁ 抗原を有する 4 種の変異株で認められ, 免疫ひなは両抗原に対する抗体を保有した。一方, 防御は両抗原を欠く 3 種の変異株では認められなかった。莢膜を保有する hi 変異株はヒアルロニダーゼで処理しても, その免疫原性と L₁ と HA-L₁ 抗原に対する抗体産生能に影響することなく, hi 変異株の病原性を不活化した。各種変異株を用いた本試験で得られた結果は, L₁ と HA-L₁ 抗原が免疫原性に関与するであろうことを示唆した著者らの従来の仮説を裏付けるものである。