Clearance of the Challenge Organisms from the Upper Respiratory Tract of Chickens Injected with an Inactivated *Haemophilus paragallinarum* Vaccine

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ABSTRACT. The challenge organisms were recovered from the chickens injected with an inactivated vaccine prepared from *Haemophilus paragallinarum* and their hemagglutination-inhibition (HI) titers were determined. Clearance of the organisms from the nasal cavity and paranasal sinus of the chickens with HI titers $\geq 1:5$ was apparent. The organisms recovered from the chickens with titers of 1:40 or higher gradually decreased in number, and the time necessary for the nasal clearance depended on the titer. The organisms were completely cleared from these chickens within 12 hr after the exposure, and they did not show any sign of coryza. Numerous organisms were recovered constantly from the chickens with titers of 1:5 to 1:20 within 6 hr after the exposure, then gradually cleared from them during 12 hr to 48 hr postchallenge, and most of them did not show any sign of coryza. In contrast, numerous organisms were constantly recovered from most of the chickens lacking the titers ($<1:5$) during 7 days postchallenge, showing proliferation of the organisms in these chickens, and most of them showed signs of severe coryza. Significant correlation was observed between the HI titer and nasal clearance of the organisms in these chickens. The inactivated *H. paragallinarum* vaccine gave solid protective immunity to the immunized chickens.—**KEY WORDS:** *Haemophilus paragallinarum*, nasal clearance, vaccine.

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Haemophilus paragallinarum causes acute upper respiratory tract infection in chickens, resulting in significant economic loss, especially by reduction in egg production. Prevention of the disease can adequately be accomplished by vaccination with the inactivated whole cell vaccine. Kato [*Jpn. J. Vet. Sci.* 32 (Suppl.): 263 (in Japanese)] first correlated the hemagglutination-inhibition (HI) antibody titers after vaccination with the protection against *H. paragallinarum* infection in chickens. From his results, the HI titer has been regarded in Japan as the protective potency given by vaccination against infectious coryza [5, 15].

For evaluating efficacy of the vaccines, prevention of clinical signs of coryza was generally employed [2, 3, 15–18, 26]. Culturing the challenge strain from the challenged chickens was also employed by Matsu-

moto and Yamamoto [14], though the recovery rates were significantly lower than morbidity. In contrast, the organisms were constantly recovered from the chickens with clinical signs of coryza, whereas rarely from those lacking the signs on 7 days postchallenge [7–12, 21, 25]. In addition, most chickens with titers of 1:5 or higher lacked the signs of coryza, whereas most chickens lacking the titers ($<1:5$) produced severe signs of coryza. Therefore, interrelation was examined among the HI antibody, signs of coryza, and clearance of the challenge organisms in the chickens.

From the results of the protection test [7–12, 21, 25], clearance of the challenge organisms from most chickens with positive HI titers ($\geq 1:5$) was apparent in 7 days postchallenge. Correlation between the HI titer and the nasal clearance of the organisms,

however, has not been clarified. The present study deals with recovery of the challenge organisms from the chickens injected with *H. paragallinarum* vaccine in relation to their HI titers.

MATERIALS AND METHODS

Bacterial strain: Encapsulated organisms of a variant designated hi was used. The variant was dissociated from strain 221 [6] of *H. paragallinarum* belonging to hemagglutination serotype HA-I [13] (agglutination serotype 1 [23]), and its properties were reported previously [7, 19].

Antigen: Organisms were grown in the S broth medium [9] at 37°C for 10 hr. The cells were collected by centrifugation at 10,000×g for 20 min, and washed once in 0.02 M phosphate-buffer, pH 7.0, containing 0.85% NaCl (PBS). The density of the cell suspension as determined with a model 6/20 spectrophotometer (Coleman Instruments) was adjusted to 1×10^{11} cells/ml, and thimerosal was added to 0.01%. The suspension referred to as nontreated (NT) antigen was used for preparation of hemagglutinin and vaccine.

Erythrocytes (RBC): Glutaraldehyde (GA, Eastman Kodak)-fixed RBC from 60-day-old line S chickens (Nisseiken Co., Yamanashi, Japan) were used for the hemagglutination-inhibition (HI) test throughout this work. The GA-fixed RBC were prepared by the method of Bing *et al.* [1].

Hemagglutination and HI tests: Hemagglutinin was prepared from the NT antigen (10^{11} cells/ml) by treating with potassium thiocyanate (KSCN) according to the method of Gaunt *et al.* [4], except the cells were incubated at 4°C for 2 hr, followed by sonication at 10 kc for 10 min (KSCN-sonicated cells) with a model 5202-T sonicator (Otake Works Co., Tokyo, Japan). The KSCN-sonicated cells were collected by centrifugation at 10,000×g for 20 min at 4°C, washed once in PBS, resuspended in PBS, and adjusted to a

concentration of 10^{11} cells/ml by spectrophotometry (KSCN-sonicated antigen) [13, 25]. The antigen was heat-labile, trypsin-sensitive, hyaluronidase-resistant, active against GA-fixed RBC [21], and used as an HA-L hemagglutinin [13, 19, 20, 25]. The HI test was performed by the method described in a previous paper [13].

Vaccine: The NT antigen was suspended in PBS containing 0.01% thimerosal, adjusted to a concentration of 5×10^9 cells/ml by spectrophotometry, and finally added to 900 µg/ml of Al(OH)₃ as adjuvant.

Injection and challenge: Vaccine (0.5 ml/chicken) was injected intramuscularly into 5-week-old specific-pathogen-free chickens (Nisseiken). Serum samples were obtained for titration of the HI antibody titers 20 days after the injection (1 day before the challenge). The chickens were divided into seven groups. Immunologic tests were performed as follows: protective immunity of the vaccine was determined by intranasal challenge with 221-hi variant [7, 19] [at least 10^8 colony-forming units (CFU)/0.2 ml/chicken] 3 weeks after the injection. The chickens were euthanatized in 1, 3, 6, 12, 24, and 48 hr and 7 days after the challenge. At necropsy, the chickens were examined for severity of clinical signs of coryza, runny nose and facial edema, according to the criteria described previously, and those with severe or moderate signs [19] were regarded as clinically positive. At necropsy, samples obtained on cotton swabs (approximately 0.01 g/sample) from the paranasal sinus or the nasal cavity of each chicken were directly streaked on an S agar plate [9]. In some cases, in which proliferation of bacteria was anticipated, samples obtained on cotton swabs were each dissolved well in 0.5 ml of S broth medium, and 0.01 ml of the diluted sample was streaked on an S agar plate. After 24-hr incubation at 37°C, colonies grown on the medium were counted, and recovery of the organisms/0.01 g of sample was express-

Fig. 1. Recovery of the challenge organisms from the nasal cavities of the chickens injected with *H. paragallinarum* vaccine.

- a) The hemagglutination-inhibition (HI) titer was determined in 20 days postinjection (1 day before challenge).
- b) Noninjected control chickens.
- c) Data are shown as an average of the organism numbers recovered from the chickens positive for the isolation.

The data are the results of the same chickens shown in Table 1.

ed as follows: – (no recovery), + (<10 CFU), ++ (<10⁴ CFU), and +++ (≥10⁴ CFU). For control, noninjected chickens were also challenged.

RESULTS

The challenge organisms were recovered from injected and noninjected control chickens in 1 to 48 hr postchallenge at various intervals (Figs. 1 and 2 and Table 1). The organisms were recovered in various numbers from all the chickens with various HI antibody titers an hour postchallenge. The number of the organisms recovered from the chickens with titers of 1:40 or higher gradually decreased, and the time necessary for the nasal clearance largely depended on the HI titer. The organisms were completely cleared

from these chickens within 12 hr after the exposure without any incidence of proliferation of the organisms and appearance of clinical signs of coryza in these chickens (Table 2). Numerous organisms were recovered from the chickens with titers of 1:5 to 1:20 within 6 hr postchallenge, but the organisms recovered were gradually decreased in number during the experimental period, and most of these chickens did not show any signs of coryza. The time for the nasal clearance also depended on the titer of each chicken. In contrast, numerous organisms were recovered constantly from most of the chickens lacking the titers (<1:5) during the period, and most of them showed severe signs of coryza in 12 hr or later postchallenge.

Correlation between the HI antibody titer

Fig. 2. Recovery of the challenge organisms from the paranasal sinus of the chickens injected with *H. paragallinarum* vaccine.

a) Noninjected control chickens.

The data are the results of the same chickens shown in Table 1.

Table 1. Recovery rate of the challenge organisms from the chickens injected with *H. paragallinarum* vaccine in 1, 3, 6, 12, 24, and 48 hr postchallenge

HI ^{a)} titer against 221-HA-L hemagglu- tinin	1 hr		3 hr		6 hr		12 hr		24 hr		48 hr	
	Nasal cavity	Para- nasal sinus	Nasal cavity	Para- nasal sinus	Nasal cavity	Para- nasal sinus	Nasal cavity	Para- nasal sinus	Nasal cavity	Para- nasal sinus	Nasal cavity	Para- nasal sinus
Injected												
≥1:320	4/4 ^{b)}	4/4	1/4	2/4	0/3	0/3	0/2	0/2	0/3	0/3	0/2	0/2
1:160	4/4	4/4	2/4	2/4	0/4	0/4	0/2	0/2	0/2	0/2	0/2	0/2
1:80	4/4	4/4	4/4	4/4	2/4	2/4	0/2	0/2	0/4	0/4	0/4	0/4
1:40	4/4	4/4	2/2	2/2	2/4	2/4	0/4	0/4	0/4	0/4	0/4	0/4
1:20	4/4	4/4	4/4	4/4	2/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4
1:10	4/4	4/4	2/2	2/2	3/4	4/4	1/4	2/4	2/4	3/4	0/4	0/4
1:5	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	4/4
<1:5	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Total	32/32 (100%)	32/32 (100%)	23/28 (82%)	24/28 (86%)	17/31 (55%)	21/31 (68%)	9/26 (35%)	10/26 (38%)	10/29 (34%)	11/29 (41%)	7/28 (25%)	8/28 (29%)
Control												
<1:5	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

a) The hemagglutination-inhibition (HI) titer was determined in 20 days postinjection (1 day before challenge).

b) Number of chickens positive for isolation/number of chickens employed.

The data are the results of two tests conducted at different times. The chickens were injected with the vaccine (0.5 ml/chicken) at 5 weeks of age, challenged (strain 221—at least 10^8 CFU/0.2 ml/chicken) intranasally at 8 weeks of age, and euthanatized at 9 weeks of age.

Table 2. Appearance of signs of coryza in chickens injected with *H. paragallinarum* vaccine in 1, 3, 6, 12, 24, and 48 hr postchallenge

HI titer against 221-HA-L-hemagglutinin	Number of chickens with clinical signs of coryza/number of chickens employed ^{a)}					
	1 hr	3 hr	6 hr	12 hr	24 hr	48 hr
Injected						
≥1:320	0/4	0/4	0/3	0/2	0/3	0/2
1:160	0/4	0/4	0/4	0/2	0/2	0/2
1:80	0/4	0/4	0/4	0/2	0/4	0/4
1:40	0/4	0/2	0/4	0/4	0/4	0/4
1:20	0/4	0/4	0/4	0/4	0/4	0/4
1:10	0/4	0/2	0/4	0/4	0/4	0/4
1:5	0/4	0/4	0/4	0/4	1/4	1/4
<1:5	0/4	0/4	0/4	3/4	3/4	2/4
Total	0/32	0/28	0/31	3/26 (12%) ^{b)}	4/29 (14%) ^{b)}	3/28 (11%) ^{b)}
Control						
<1:5	0/6	0/6	0/6	2/6	6/6	6/6

a) The same chickens shown in Table 1.

b) Morbidity determined by appearance of clinical signs of coryza. All the diseased chickens showed moderate to severe signs of coryza.

and the protective immunity given by vaccination was investigated in 7 days postchallenge. Of 231 chickens injected with the vaccine, 203 (88%) produced the HI titers ranging from 1:5 to ≥1:320. The challenge organisms were completely cleared from all the chickens with titers of 1:20 or higher, whereas those with titers of 1:10 or higher completely lacked any sign of coryza. The protection rate defined by morbidity (prevention of clinical signs of coryza) was generally higher than the rate of nasal clearance (no recovery of the organisms).

DISCUSSION

Analytic studies [19, 20, 24] on the antigen structure of *H. paragallinarum* organisms belonging to hemagglutination serotype HA-I [13] (agglutination serotype 1 [23]) showed that the organisms had heat-labile, trypsin-sensitive, hyaluronidase-resistant HA-L hemagglutinin [25] and L agglutinin [24]. The antigens HA-L and L, components of

outer membrane of the bacterial cell [20], were variant specific among the seven variants dissociated from the serotype HA-I strains [19]. The results of *in vivo* protection test showed that both antigens HA-L and L are responsible for protective immunity against the organisms [7–12, 21, 25], although antigenic similarity between the two somatic antigens has remained to be clarified. Recovery of the challenge organisms from the chickens injected with an inactivated *H. paragallinarum* vaccine was tested in relation to their HI titers with HA-L antigen as a hemagglutinin for the HI test.

Nasal clearance of the challenge organisms of *H. paragallinarum* from the chickens immunized with an inactivated vaccine was apparently observed at an early stage after the challenge, if the chickens had positive (≥1:5) HI antibody titers against the HA-L hemagglutinin [25]. In addition, the period of time for the clearance largely depended on the HI titer. The patterns of the nasal clearance

Table 3. Correlation between HI titer and protection of the chickens injected with *H. paragallinarum* vaccine against intranasal challenge with strain 221 on day 7 postchallenge

HI titer against 221-HA-L hemagglutinin	Number of chickens with HI titer ^{a)}	Number of chickens with signs of coryza	Number of chickens positive for isolation of the organisms
Injected			
<1:5	28	14 (50%) ^{b)}	18 (36%) ^{c)}
1:5	22	2 (91%)	5 (77%)
1:10	29	0 (100%)	2 (93%)
1:20	29	0 (100%)	0 (100%)
1:40	22	0 (100%)	0 (100%)
1:80	32	0 (100%)	0 (100%)
1:160	34	0 (100%)	0 (100%)
≥1:320	35	0 (100%)	0 (100%)
Total	231	16 (93%)	25 (89%)
Control			
<1:5	15	15 ^{d)}	15 ^{d)}

a) The titer was determined in 20 days postinjection (1 day before challenge).

b) Protection rate defined by morbidity (prevention of clinical signs of coryza).

c) Protection rate defined by nasal clearance (no recovery of the organisms).

d) All the noninjected control chickens were infected.

The data are the results of three tests conducted at different times. The chickens were injected with the vaccine (0.5 ml/chicken) at 5 weeks of age, challenged (strain 221=at least 10^8 CFU/0.2 ml/chicken) intranasally at 8 weeks of age, and euthanatized at 9 weeks of age.

were divided into two types. The challenge organisms gradually decreased in number, and the organisms were completely cleared within 12 hr after the exposure from the chickens with titers of 1:40 or higher without any incidence of *in vivo* proliferation of the organisms. Therefore, the clearance might occur before proliferation of the organisms in these chickens, even though $>10^8$ CFU/chicken of the organisms were administered intranasally. The number of the organisms recovered from the chickens with titers of 1:5 to 1:20 were very similar for 6 hr postchallenge, then gradually decreased, and the organisms were finally cleared from them within 48 hr postchallenge depending on the HI titer. The organisms may proliferate slightly in these chickens at an early stage after the challenge; however, the power of the nasal clearance overcomes proliferation of the organisms. In contrast, proliferation of the organisms in both the injected chickens with titers of 1:5 and noninjected control chickens lacking the HI titers was apparent. The organisms in large numbers persisted in the upper respiratory tracts of these chickens even in 7 days postchallenge. Nasal clear-

ance of the proliferated organisms from these chickens may occur after the HI antibody is produced, as previously suggested by Sawata and Kume [19]. Significant correlation was observed between the HI titer and nasal clearance of the organisms from or suppressed proliferation of the organisms in the immunized chickens. At least a titer of 1:5 is necessary for the nasal clearance of *H. paragallinarum*.

The encapsulated hi variant [7, 19] of *H. paragallinarum* produced signs of coryza in the chickens lacking any detectable antibody against the organisms in 2 days after intranasal inoculation of the organisms of $>10^8$ CFU/chicken intranasally [19]. As shown herein, signs of coryza were recognized first in 12 hr postchallenge in some noninjected control chickens lacking the HI titer, and most of the injected chickens including those having titers lower than 1:5 produced the signs within 24 hr after the exposure. Numerous challenge organisms were generally recovered from these chickens even 7 days postchallenge, showing *in vivo* proliferation of the organisms. In contrast, most of the injected chickens with various HI antibody

titers did not produce any signs of coryza, and the organisms were cleared rapidly from these chickens within 48 hr after the exposure. Proliferation and persistence of the organisms in the upper respiratory tract of the chickens may be essential for production of signs of coryza. A titer of at least 1:10 may be necessary for prevention of the signs.

Previously, Sawata and Kume [19] suggested that appearance of signs of coryza in chickens is related to a certain substance in the capsule or that specifically produced by the encapsulated organisms of *H. paragallinarum*. Numerous challenge organisms persisted in the upper respiratory tracts of the chickens with HI titers of 1:5 during the first 24 hr after the exposure; signs of coryza developed only in some of these chickens. It is unlikely, therefore, that any capsular substance is responsible for developing signs of coryza. A certain toxic substance responsible for the signs may be released from the encapsulated organisms during proliferation.

It is well known that the antigens L and HA-L of *H. paragallinarum* are important in protection of chickens [7-12, 21, 25]. More recently, antigens L and HA-L were shown *in vitro* as a target for the bactericidal activity of the immune chicken serum having the HI antibody against the HA-L hemagglutinin and the agglutinin against L agglutinin [22]. From the results obtained both *in vivo* and *in vitro* studies, antigen HA-L or L or both seems to be a protective antigen of *H. paragallinarum*. As shown herein, the HI titer-dependent active protection against the intranasal challenge can be conferred on the chickens with an inactivated vaccine prepared from *H. paragallinarum*. However, it is not known whether this protection is dependent only on the level of the HI titer, nor, if so, how it acts.

An inactivated *H. paragallinarum* vaccine gave solid protective immunity to chickens. The nasal clearance of the challenge organisms from the chickens at an early stage of the

infection by the action of the HI antibody is evident. The clearance largely depended on the HI titer. Immunization of chickens with a vaccine of a high quality, therefore, is essential to control the disease caused by *H. paragallinarum*.

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要 約

Haemophilus paragallinarum 血清型 HA-1 221 株不活化ワクチン前処置ひなにおける攻撃菌の消長: 久米勝巳・澤田 章¹⁾・中井豊次¹⁾ (北里研究所, ¹⁾北里研究所附属家畜衛生研究所) — *H. paragallinarum* (Hpg) 不活化ワクチン前処置ひなから経時的に攻撃菌の消長をみた。攻撃時に赤血球凝集抑制 (HI) 抗体価 1:40 以上のひなでは、攻撃直後から菌数の減少が認められ、攻撃後12時間までに完全に消失した。菌消失までの時間は HI 抗体価が高いほど短かく、これらのひなでは菌増殖はみとめられなかった。1:5~1:20 の HI 抗体保有鶏では、攻撃後6時間までは多数の菌が回収されたが、以後菌数は減少し攻撃後48時間までにほぼ消失した。一方、HI 抗体を欠くひなでは、攻撃後7日目まで常時多数の菌が回収された。