Properties of Heat-Stable Antigen in the Culture Supernate of *Haemophilus paragallinarum*

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Abstract. To analyze the antigenic component of *Haemophilus paragallinarum* (Hpg), serological and immunological properties of a heat-stable antigen in the culture supernate of Hpg were studied. At least two different antigens were found in the culture supernate of various Hpg strains by the agar gel precipitation (AGP) test. They were separated into two fractions by gel filtration. The heat-stable antigen was eluted at void volume by gel filtration with Sepharose 6B and 2B, but not any other. The heat-stable antigen did not lose antigenicity when it was heated at 121°C for 30 minutes. The AGP test revealed that the heat-stable antigen was not type-specific to any Hpg serotype. Moreover, lipopolysaccharide (LPS), which had been isolated from Hpg cells by Westphal's methods, was found to have antigenicity common to Hpg serotypes. In the AGP test, the heat-stable antigen showed an identity reaction to the LPS product. Neither heat-stable antigen in the culture supernate nor LPS antigen of Hpg strain 221 presented a protective effect when injected into chickens, or agglutinated chicken red blood cells. Furthermore, no measurable hemagglutination-inhibition antibody was found in chickens immunized with both antigens. These results suggested that heat-stable antigen in the culture supernate of Hpg might be composed of LPS.

Some different antigens were found in sonicated cells of *Haemophilus paragallinarum* (Hpg) in the agar gel precipitation (AGP) test [8, 14, 15]. Recently, Iritani [3] reported that at least two different precipitins existed in the supernate of trypsinized Hpg cells. The antigens in the cells could be divided into at least two types according to their reaction to heating. One was heat-stable and the other not [15].

In their previous study, the authors reported that antigens in the culture supernate had a protective effect against Hpg infection and were composed of at least two different precipitinogen [6]. These soluble antigens are presumed to be important substances for the serodiagnosis of Hpg infection and the preparation of Hpg bacterin. Their serological or immunological proper-

ties, however, had not been well clarified.

The present investigation is a part of a series of studies on antigen analysis of the Hpg component. This paper describes some of the properties of heat-stable antigen in the culture supernate of Hpg.

Materials and Methods

Bacterial strains: Table 1 lists the strains of Hpg used. Hpg strains 1645 and 2403 were kindly supplied by Dr. Hinz, of the Institute of Poultry Disease, Hanover, West Germany [2], and Hpg strains 0083, 221, 288, F [10], and Tamasato by the National Institute of Animal Health, Tokyo, Japan. Two other strains were recently isolated at the authors' laboratories from chickens affected with infectious coryza. The chickens had been vaccinated sufficiently with commercial Hpg bacterin prepared from strain 221 alone. Strain SI among the authors' isolates was cross protected to strain Modesto, but was not strain 221.

Broth culture: Hpg was inoculated into 10 ml

of chicken meat infusion broth supplemented with 0.5% chicken serum, and then incubated at 37°C for 24 hours. Two milliliters of the starter culture was inoculated into 2,000 ml of Kato's broth prepared as previously described [5]. Cells and supernate were separated by continuous-flow centrifugation at 10,000 rpm at 4°C at a flow rate of 4 l/hr after incubation at 37°C for 48 hours.

Extraction of antigen in culture supernate: The supernate was filtered through a 0.2 μ m Sartorius membrane filter and concentrated to about 150-fold of the initial supernate using a membrane filter apparatus (Millipore; 100,000 molecular weight). The concentrated materials were dialyzed in distilled water and stored at -20°C until use (crude antigen). The crude materials were separated by gel filtration on Sepharose 6B and 2B [column (30 $\times$ 730 cm) at pH 7.8]. The column was eluted with 0.1 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl at a flow rate of about 10 ml per 25 minutes. Portions of the fractions were used for component analysis of neutral sugar by the phenol-sulfuric acid methods (490 nm) [1] and of protein by absorbance at 280 nm using Uvicord II (LKB, Brama, Sweden). The heat-stable antigen fraction was concentrated by lyophilization after dialysis, and ultracentrifuged at 110,000 $\times$ G for 2 hours for further purification. The sediments were dissolved in Tris-HCl buffer and refiltrated on Sepharose 6B gel (partially purified antigen).

Westphal-type lipopolysaccharide: The harvested cells were washed three times and resuspended in 2 M NaCl. The packed cells were washed more than twice with phosphate-buffered saline solution (PBSS), pH 7.0, after incubation overnight at 37°C. The suspensions were trypsinized three times by the methods described previously [3]. The resulting sediment was used for preparation of Westphal-type lipopolysaccharide (W-type LPS). The trypsinized cells were washed three times with PBSS and suspended in distilled water. The suspension was heated to 68°C in a water bath and an equal volume of 90% phenol heated to 68°C was added. The suspension was incubated in a water bath with shaking for 30 minutes. Next, it was centrifuged at 2,000 $\times$ G at 4°C for 10 minutes. The aqueous phase was removed and dialyzed. The phenol phase was reextracted. W-type LPS was collected by ultracentrifugation at 110,000 $\times$ G for 2 hours.

Serological test: AGP test was performed by the methods described previously [8]. Hemagglutination-inhibition (HI) titer was determined by the methods described previously [13] by using 4 hemagglutinating units of trypsin-sensitive hemagglutinin which termed type 1 hemagglutinin [17]. Slide

agglutination (AGG) test and preparation of the antigen were carried out by the methods described previously by using trypsinization [7]. These titers were read as the highest dilutions of serum or the fraction. Hemagglutination titer was measured by the methods described previously [5].

Immune sera: Thirty-day-old White Leghorn chickens were used. They had been reared at the authors' laboratory and were free from specific pathogens, including Hpg. Groups of five chickens each were injected intravenously three or four times at one-week intervals with W-type LPS and partially purified antigens of Hpg strain 221. Sera were collected from the immunized chickens 7 days after the last injection and then pooled after determination of AGP, HI and AGG titers. Convalescent sera were obtained from the chickens 4 weeks after the artificial infection with various Hpg strains.

Challenge test: Immunized and non-immunized chickens were inoculated, by nasal instillation, with 0.5 ml of suspension of live Hpg organisms containing 10^8 to $10^{8.5}$ colony forming units/ml. Clinical respiratory signs, appearance of nasal discharge and swelling of the face were recorded daily. The chickens were killed 7 days after challenge and observed for gross lesions in the infraorbital sinuses. Any exudate from the infraorbital sinuses was streaked with cotton swabs onto blood agar, which was incubated in 5% CO₂ at 37°C for 48 hours for Hpg isolation [6].

Results

Gel filtration of antigen in culture supernate: At least two different antigens were detected in all the crude antigens from various Hpg strains by the AGP test. Fig. 1 shows the gel filtration on Sepharose 6B of the crude antigen of strain 221 with a tracing at both 280 and 490 nm. The crude antigen not exposed to heating was divided into two fractions by the AGP test with homologous convalescent Hpg serum. However, when the antigen was heated at 100°C for 20 minutes, it produced only one precipitin eluted at void volume by the AGP test. The heat-stable antigen lost no antigenicity after additional heating at 121°C for 30 minutes. As shown in Fig. 2, the elution profile of the two antigens of strain 2403 was similar to that of strain 221. The same findings were

Fig. 1. Sepharose 6B gel filtration on culture supernate of strain 221 of *Haemophilus paragallinarum*.

Fig. 2. Sepharose 6B gel filtration on culture supernate of strain 2403 of *Haemophilus paragallinarum*.

obtained from antigen in the culture supernate of various Hpg strains. When the heat-stable antigen of strain 221 was filtered with Sepharose 2B, it was eluted at void volume.

When the heat-stable fraction was collected and concentrated to about ten times

the initial volume, heat-labile antigen was detected from this fraction by the AGP test. The contamination of the heat-stable fraction with heat-labile antigen, however, could be avoided by refiltration on the gel after ultracentrifugation.

Fig. 3. Agar-gel precipitation test of convalescent chicken serum to various strains of *Haemophilus paragallinarum*.

G, Partially purified heat-stable antigen of strain 221; A-F, Convalescent chicken sera (A: to strain 221. B: 0083. C: Tokai. D: S1. E: F. F: 288).

Table 1. Serotype and source of strains of *Haemophilus paragallinarum* used

Name of strain	Source	Serotype	
		Page	Kato
0083	California, USA	A	I
1645	West Germany	A	×
2403	West Germany	B	×
221	Tokyo, Japan	A	I
288	Gunma, Japan	×	II
F	Tokyo, Japan	×	III
Tamasato	Japan	×	II
Tokai	Mie, Japan	×	×
S1	Gifu, Japan	C	×

×: Unknown.

Serologic properties of partially purified antigen by AGP test: Convalescent chicken sera of strains 221, 288, F, 0083, Tokai and S1 had antibody against partially purified antigen of strain 221, as evidenced by the appearance of one line (Fig. 3). When strain 221 antigen in the center well in Fig. 3 was replaced by partially purified antigen of various strains listed in Table 1, one corresponding AGP line was also found in each of the convalescent sera. As can be seen in Fig. 4, one corresponding line was found in

Fig. 4. Agar-gel precipitation test of heat-stable extracellular antigen of strains 221 and 2403 of *Haemophilus paragallinarum*.

A, Convalescent chicken serum to strain 2403; B, Partially purified heat-stable antigen of strain 221; C, Partially purified heat-stable antigen of strain 2403.

Fig. 5. Agar-gel precipitation test of heat-stable extracellular antigen and lipopolysaccharide of *Haemophilus paragallinarum*.

A, Convalescent chicken serum to strain 221; B, Lipopolysaccharide of strain 221; C, Partially purified heat-stable antigen of strain 221.

partially purified antigens between Hpg strains 221 and 2403 which were different serotypes.

Fig. 5 shows AGP lines between partially purified and W-type LPS antigens of strain 221. One AGP line was observed with contiguity between two antigens (Fig. 5). It did not disappear when both antigens had been heated at 121°C for 30 minutes. The line found by W-type LPS of 221 strain was also identical with that of various heat-stable antigens from strains 288, F, Tamasato, Tokai, S1, 1645 and 2403.

Table 2. Immune effects of heat-stable antigen in culture supernate and Westphal-type lipopolysaccharide (W-type LPS) antigen of *Haemophilus paragallinarum* strain 221

Immunized with	No. of chickens used	Geometric mean antibody titer at challenge			Results of challenge		
		HI*	AGP**	AGG†	No. of positive chickens		
Heat-stable antigen	5	<1:2 (<1:2-<1:2) ‡	1:6 (1:4-1:32)	1:9 (1:5-1:20)	5	5	5
W-type LPS	5	<1:2 (<1:2-<1:2)	1:4 (1:2-1:16)	1:165 (1:64-1:512)	5	5	5
None	10	<1:2	<1:1	<1:1	10	10	10

* Hemagglutination-inhibition titer.

** Agar-gel precipitation titer to homologous antigen.

† Slide agglutination titer by trypsinized whole cells of a homologous strain.

‡ Minimum to maximum titers.

Immune effect: Table 2 presents the protective effect to W-type LPS and partially purified antigens of strain 221 to homologous Hpg infection. AGP and AGG antibodies were found in the sera of chickens immunized with both antigens of strain 221 at challenge, but no HI antibody was detected in any of the sera. Moreover, no measurable hemagglutinating activity was observed in 1 mg of W-type LPS or 2 mg of partially purified antigens. No chickens were protected against challenge with homologous similar to the non-immunized chickens group.

Discussion

The present study showed that heat-stable antigen in the culture supernate of Hpg was resistant to heating at 121°C for 30 minutes, as well as W-type LPS of Hpg was. Furthermore, it was revealed that a cross reaction occurred between heat-stable antigen and W-type LPS in the AGP test. The molecular weight of heat-stable antigen seemed to be about several millions according to gel filtration. Generally, it is shown that bacterial LPS was eluted from cells to the culture supernate by autolysis. The results of the present study seem to show that the heat-

stable antigen in the culture supernate may be composed of bacterial LPS.

Recently, Sawata et al. [16] conducted analysis of antigenic structure between serotypes 1 and 2 of Hpg by the AGG test. They suggested that one of the antigens common to the serotypes might be heat-stable and trypsin-resistant. In the present study, the heat-stable antigen in the culture supernate was proved to be common to the Hpg serotypes by the AGP test. Probably, the heat-stable and trypsin-resistant antigen reported by Sawata et al. may be bacterial LPS shown in the present study.

Hpg strain 221 agglutinate chicken red blood cells [9]. The Hpg-HI response of chickens following vaccination with Hpg-killed bacterin appears to be correlated with protection against the infection [9, 13]. Furthermore, the purified hemagglutinin of Hpg strain 221, which was composed of protein and sugar and heat-labile, seems to be a protective component of Hpg [4]. A previous study found that antigen in the culture supernate of Hpg lost its protective effect by heating [6]. On the other hand, Kume et al. found no cross protection between Hpg serotypes 1 and 2 [12]. The results of this study certainly indicate that

heat-stable antigen in the culture supernate does not offer protection against artificial Hpg infection. In addition, neither heat-stable antigen nor LPS agglutinated chicken red blood cells. Moreover, no HI antibody was found in chickens immunized with that antigen. As mentioned above, no type-specificity was found in either antigen. It is reasonable, therefore, to mention that LPS of Hpg causes neither hemagglutination nor protection.

Konno and Nakase [11] reported that the purified LPS of Hpg was toxic to embryonated hen's eggs. Generally, the toxic reaction by Hpg bacterin is presumed to be caused by bacterial LPS and/or aluminum-gel. The results of the present study seem to offer information available for the production of efficacious Hpg bacterin without any harmful reaction.

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要 約

ヘモフィルス・パラガリナルム培養液中に存在する耐熱性抗原の性状：入谷好一・岩城秀治・山口猛（塩野義製薬株式会社油日ラボラトリーズ）——ヘモフィルス・パラガリナルム（Hpg）の抗原性物質を解析する手始めとして，培養液中に存在する耐熱性抗原の血清および免疫性状を検討した．種々な Hpg 株の培養液には，ゲル透過で分離可能な，少なくとも 2 種類の沈降原が認められた．一つは 121°C 30 分の耐熱性で，他は 100°C 20 分の易熱性であり，前者はセファロース 2B のゲル透過で VO 値に溶出した．耐熱性抗原は，ゲル内沈降反応で菌体から抽出したリポ多糖体と一致し，Hpg 株の血清型間で共通抗原性を示した．221 株の耐熱性抗原およびリポ多糖体は赤血球凝集性を欠き，両抗原でニワトリを免疫した時，スライド凝集反応およびゲル内沈降反応に対するそれぞれの抗体が上昇した．しかし，赤血球凝集抑制抗体は検出されず，ニワトリは人工感染に対し感染防御を示さなかった．以上のことから，培養液中の耐熱性抗原はリポ多糖体であると推論された．