Local Immunization in Chicken Respiratory Tract with Killed *Mycoplasma gallisepticum* Vaccine

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Abstract. Immunity in chickens with killed organisms of *Mycoplasma gallisepticum* was evaluated according to the lesion grade of airsacculitis, the retention of *M. gallisepticum* in organs and histopathological findings. Killed *M. gallisepticum* did not provide any resistance when intramuscularly administered either alone or in admixture with adjuvants. An increased resistance to infection, however, was observed when it was administered into the respiratory tract in a form of aerosol or by intratracheal injection. A partial immunity was maintained at a significant level for at least 3 months. These results suggest that protection of chickens against *M. gallisepticum* infection may be possible when the killed vaccine is applied directly to the lower respiratory tract of the chickens. The suggestion is supported by the histopathological findings of specimens collected from immunized birds. Little correlation was seen between serological serum titer and resistance to challenge.

Immunity in chickens to *Mycoplasma gallisepticum* infection was demonstrated early by Nelson [9]. He observed that chickens recovered from chronic coryza induced by intranasal inoculation with the tracheal exudate from diseased chickens were resistant to secondary exposure. Immunity of chickens after natural infection [4, 13, 14] or experimental infection [1, 2, 5, 8] with *M. gallisepticum* was proved later by a number of investigators. These chickens were recovered from the infection and showed immunity when exposed again to the homologous strain.

On the other hand, some workers [1, 2, 7, 12] attempted to obtain protection with

the killed organism either as plain vaccine or as vaccine with adjuvant and found only poor effects, except Warren et al. [15] who stated that an inactivated vaccine had increased the resistance of 1-day-old chicks to air-sac inoculation with the homologous strain. Soběslavský et al. [11] reported that *M. gallisepticum* was adsorbed by the epithelial cells of the chicken respiratory tract.

The present experiment was performed to observe an effect of local application of killed organisms, with an expectation that there might be some increased stimulation and biologic reaction to the establishment and proliferation of organisms having in-

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vaded the respiratory tract.

Materials and Methods

Chickens: The broiler chickens used in the experiment were supplied by the Japan Shaver Grand Parent Poultry Farm, Inc. They were free from *M. gallisepticum* and antibody against it.

Mycoplasma strain: *M. gallisepticum* strain S6 was obtained from Dr. H. E. Adler. It had passed 17 times in Mycoplasma liquid medium at the authors' laboratory. The virulent strain, S6-K, was developed from strain S6 at this laboratory by three passages in chick embryos inoculated into the yolk sac which obtained from an SPF flock of fowls free of mycoplasmas and twelve passages in chickens by intratracheal inoculation. The isolate was tested for purity biologically, biochemically and serologically. Its virulence was tested in chickens by intratracheal or aerosol inoculation of the diluted culture. The organisms were lyophilized for storage and thawed before use. Strain S6-K was used both for the preparation of killed vaccine and for challenge inoculation.

Culture media: Mycoplasma broth or Mycoplasma agar (The Kitasato Institute, Tokyo, Japan) was enriched with 10 per cent inactivated horse serum and 0.1 per cent glucose. If necessary, approximately 200 units of penicillin G and 250 μ g of thallium acetate were added per ml of medium. For the preparation of killed vaccine used for immunization, inactivated horse serum in the liquid medium was replaced by inactivated chicken serum.

Preparation of killed vaccine: Ten liters of the liquid medium was inoculated with 250 ml of fresh 48-hour culture of *M. gallisepticum* that had been subjected to five passages in the liquid medium containing chicken serum under aerobic conditions at 37°C for 2 days each. The culture was centrifuged at 13,000 rpm for 30 minutes at 4°C. The deposited cells were resuspended in 0.01 M phosphate buffered saline (pH 7.2) to give a cell concentration of 2.0×10^{10} CFU per ml (1/20th of the original volume). Formalin was added to the cell suspension at a final concentration of 0.01 per cent to kill the cells. The cultivation test revealed that cell killing had been completed in the preparation.

The adjuvants used as immune promoters were Freund's complete adjuvant (Difco), magnetic iron oxide (Pfizer Co.) at 20 mg per ml, sodium alginate (Kimitsu Chemical Industry Co., Ltd.) at 20 mg per ml, and aluminum hydroxide gel at 4.4 mg per ml, either alone or with inactivated *Hemophilus gallinarum* at 2.6×10^{10} per ml. The adjuvant was mixed with an equal volume of killed cell suspension of

M. gallisepticum.

Titration of fifty per cent infectious dose (ID_{50}): Groups of approximately ten normal 42-day-old chickens each were infected with serially diluted cultures by aerosol spraying. All the chickens were sacrificed 2 weeks later. The trachea, lung, and air sac were aseptically collected from them and emulsified. The tissue supernatant fluid prepared from the resulting emulsion was cultivated quantitatively at 37°C for 10 days. ID_{50} was determined to be 2.0×10^3 CFU on the proportion of positive birds.

Vaccination of chickens:

(1) To examine the effect of the adjuvant and the route of administration for immunization, 10-day-old chickens were administered intramuscularly with 1 ml of either plain or adjuvant vaccine, and intratracheally with 0.5 ml of killed vaccine. In the case of sodium alginate vaccine, chickens were injected simultaneously with 1 ml of calcium chloride (8.3 mg per ml), as described by Scherr et al. [10], at the same site. The immunization was carried out 3 times at 10 days' intervals. Twelve days after the last immunization, all the immunized birds and the unimmunized controls were challenged intratracheally with 3.2×10^5 CFU of the homologous strain. Two weeks after the challenge, all the chickens were sacrificed. Formalin-killed plain vaccine and aerosol challenge infection were employed in all the following studies.

(2) An experiment was carried out to compare routes of inoculation with plain vaccine. In it 10-day-old chickens were treated in the following manner; intratracheal injection with 0.5 ml, intranasal inoculation with 0.25 ml, or inhalation with vaccine aerosol. The aerosol administration was conducted in a vinyl isolator (product of the Clea Japan, Inc.) to measure the inhalation volume of 10 chickens per minute. Into another collapsed 150-liter vinyl isolator the killed cell suspension was sprayed by a nebulizer (product of the Toyoshima Co.) having a capacity of 0.25 ml per minute under the pressure of 0.7 kg per square centimeter. When the pre-estimated volume of aerosol was placed in the second isolator, the two isolators were connected with each other through a tunnel and the chickens were transferred into the second isolator.

Spraying was continued further, until the chamber content reached a certain number of killed organisms in the aerosol. This number was calculated from the spraying period of time and the respiratory volume. One dose of vaccine per chicken for aerosol immunization was controlled in such manner as to contain 5.0×10^9 cells. The vaccination was carried out three times at 10 days' intervals.

Each immunized chicken was challenged by aerosol with a dose of 7.4×10^5 , 7.4×10^4 or 7.4×10^3 CFU 2 weeks after the last immunization and sacrificed another 2 weeks later.

(3) Then a contagious infection test was conducted. In it, chickens were immunized with vaccine aerosol two or three times at 10 days' intervals, the dose per chicken being 5.0×10^9 CFU each time. The first immunization for a two-dose group was performed to 10 twenty-day-old birds, and that for a three-dose group to 10 ten-day-old ones. Twelve days after the final immunization, all the immunized and 9 non-immunized chickens were allowed to live together with 10 chickens which had been infected by aerosol with 2.3×10^5 CFU of *M. gallisepticum*. Four weeks later, they were sacrificed.

(4) An experiment was carried out to inquire into a relationship between vaccine dosage and protection. Groups of 10 chickens each were immunized with 5.0×10^9 , 1.0×10^9 or 2.0×10^8 CFU per chicken two or three times at 10 days' intervals. The immunization schedule established was the same as mentioned previously. Two weeks after the final immunization, all the immunized and 10 control chickens were challenged with 2.0×10^4 CFU per chicken. In each group, a half of the birds was sacrificed 2 weeks after the challenge inoculation and the other half 2 weeks later.

(5) An experiment was conducted to examine the persistence of immune state. Vaccination was performed in the same manner as described previously. The dose per chicken was 5.0×10^9 or 1.0×10^9 CFU when 3 doses were used at 10 days' intervals and 5.0×10^9 CFU when 2 doses were used. Each of 3 immunized and one control groups was composed of approximately 30 chickens. In each group, one-third of the birds was challenged with 6.9×10^3 CFU 1 month after the last immunization, another one-third with 5.5×10^3 CFU 3 months after, and the remaining birds were with 2.8×10^4 CFU 6 months after. All the birds were sacrificed 2 weeks after the challenge.

Scoring: The evaluation of immune effects was made by the macroscopic lesion score of the air sacs, the isolation of organisms from the trachea, lungs and air sacs, and histological findings. The score of each chicken was obtained as an average value of the degree of lesions in four air sacs. The degrees of lesions were expressed as follows: 0, normal; 1, thickening of air sac; 2, focal lesions; 3, formation of 1 or a few caseous lumps; and 4, formation of many or overwhelming caseous lumps.

Quantitation of *M. gallisepticum*: Trachea, lung and air sac were collected aseptically from chickens

sacrificed, ground in a glass homogenizer, and made to 10% (w/v) emulsion in liquid medium. The organ emulsion was centrifuged at 1,500 rpm for 15 minutes. The resulting supernatant fluid was diluted serially ten-fold in liquid medium, and 0.01 ml of each dilution was inoculated in duplicate on to solid medium. The plates were incubated at 37°C for 10 days, until the number of colonies was counted.

Serologic test: Sera were collected from immunized and control chickens at different intervals before and after the immunization and after the challenge inoculation. The tube agglutination (TA) test was performed by the method of Ando et al. [3], and the hemagglutination-inhibition (HI) test by the procedure of Kuniyasu et al. [6].

Histological examination: Specimens were taken from trachea, lung and air sac, and fixed in 10% formalin. Routine paraffin sections were made and stained with hematoxylin and eosin.

Results

Effects of adjuvants and routes of vac-cinal administration: The results obtained are shown in Table 1. *M. gallisepticum* organisms were isolated from the organs of all chickens. The mean score of air sac lesions indicates that any significant protection was not provided by the intramuscular route, regardless of the kind of vaccine, while it was markedly low when the intratracheal route was employed. TA antibody titers of sera obtained from immunized birds immediately before challenge ranged from 29.3 to 96.0, whereas HI antibody did not developed. In sera obtained 2 weeks after challenge, TA and HI antibody titers were significantly high in all the birds, except the uninfected controls.

Histopathologically, the wall of the air sac of the normal chicken usually showed only slight inflammatory cell infiltration (Fig. 1).

Four to six weeks after the last immunization, histological examination revealed no difference among normal chickens and immunized ones which had not been challenged. However, the wall of the air sac

Table 1. Effects of various adjuvants and routes of administration of inactivated *M. gallisepticum* vaccine in broiler chickens

Immunization (3 doses at 10 days' intervals)		Chall. dose	Number infected/Total	Mean score of air sac lesion*	Mean antibody titer						
Antigen	Additive				Route	TA			HI		
						Before immun.	Before chall.	2 weeks after chall.	Before immun.	Before chall.	2 weeks after chall.
Inactivated <i>M. gallisepticum</i>	Freund's complete adjuvant	I.M.	3.2×10^5	5/5	2.75	<8	41.4	341.3	<8	<8	35
	Magnetic iron oxide			5/5	2.80	<8	29.3	298.7	<8	<8	64
	Sodium alginate			5/5	2.70	<8	48.0	170.6	<8	<8	26.6
	Inactivated <i>H. gallinarum</i> adsorbed to aluminum hydroxide			10/10	3.00	<8	40.0	341.3	<8	<8	42.6
	Aluminum hydroxide			10/10	2.70	<8	96.0	341.3	<8	<8	37.3
	None	I.T.	None	5/5	2.65	<8	30.7	278.9	<8	<8	47.1
	None			5/5	1.75	<8	30.6	202.7	<8	<8	34.7
	None			5/5	3.15	<8	<8	291.6	<8	<8	33.8
None				0.60	<8	<8	12	<8	<8	<8	

Remarks.

TA=Tube agglutination. HI=Hemagglutination inhibition. I.M.=Intramuscular.

I.T.=Intratracheal. Chall.=Challenge. Immun.=Immunization.

* Mean score of air sac lesion=Number of chickens multiplied by an average score of lesions of four air sacs divided by the number of chickens.

of chickens challenged by *M. gallisepticum* showed advanced inflammatory reaction with infiltration of numerous plasma cells, lymphocytes, and even a moderate number of polymorphonuclear leucocytes and remarkable hyperplasia of lymph follicles (Fig. 2). In the groups immunized intramuscularly, there was no histological evidence of protective effect, since the same degree of inflammatory reaction was seen in these groups as in the challenged group.

Chickens immunized with a mixture of killed vaccine and inactivated *H. gallinarum* adsorbed to aluminum hydroxide showed essentially the same inflammatory reaction of the air sac as the infected ones (Fig. 3).

On the other hand, in the group intratracheally immunized with plain vaccine, the inflammatory reaction of the air sac was reduced in severity (Fig. 4).

Comparison of immune efficacy of plain

vaccine among intratracheal, intranasal and aerosol administration: As seen in Table 2, the intranasal vaccination provided no distinct protective effect, while the intratracheal and aerosol vaccination markedly suppressed the development of the air sac lesion and/or the proliferation of the organism.

Antibody titers were at the same level before the challenge as before the immunization, except the TA antibody titer in the intratracheally immunized group. In this group, the vaccine dose was six times as large as that in any other group and the TA antibody titer after the immunization was more than four times as high as that before the immunization. HI antibody was slightly raised only in chickens challenged with the highest dose 2 weeks before. Little correlation was indicated between antibody titer and protective effect.

Almost the same pathological findings

Table 2. Comparison of immune reaction after intratracheal, intranasal and aerosol administration of inactivated *M. gallisepticum* vaccine

Immunization (3 doses at 10 days' intervals)		Chall. dose 7.4×10^{11}	Number protected*/Total	Chicken protected	Mean antibody titer					
Antigen	Route				TA			HI		
					Before immun.	Before chall.	2 weeks after chall.	Before immun.	Before chall.	2 weeks after chall.
1.0×10^{10}	I.T.	5	2/10	20%			37			12
		4	4/8	50	<8	32	20	<8	<8	<8
		3	6/8	75			25			<8
		None	8/8				<8		<8	
5.0×10^9	I.N.	5	0/8	0			44			<8
		4	0/8	0			23			<8
		3	2/8	25	<8	<8	8	<8	<8	<8
		None	8/8				<8		<8	
	Aerosol	5	2/10	20			36			12
		4	2/8	25	<8	9	16	<8	<8	<8
		3	8/8	100			9			<8
		None	8/8				<8		<8	
None		5	0/8	0			43			27
		4	0/8	0			43			<8
		3	0/8	0	<8	<8	24	<8	<8	<8
		None	10**				<8		<8	

Remarks.

TA=Tube agglutination. HI=Hemagglutination inhibition. Chall.=Challenge. Immun.=Immunization.

I.T.=Intratracheal. I.N.=Intranasal.

* Number of chickens showing both negative isolation of *M. gallisepticum* and a score of less than 2.0

** Not challenged.

Table 3 Immune reactions in contagious infection test of chickens immunized in respiratory tract with inactivated *M. gallisepticum* vaccine

Immunization (at 10 days' intervals)	Antigen	Infection*	Number protected/ Total	Chickens protected	Mean antibody titer					
					TA			HI		
					Before immun.	Before chall.	4 weeks after contag.	Before immun.	Before chall.	4 weeks after contag.
5.0×10^9 3 doses			10/10	100%	<8	<8	9.6	<8	<8	<8
5.0×10^9 2 doses		Contagious infection with challenge group	10/10	100	<8	<8	<8	<8	<8	<8
None			6/9	66.7	<8	<8	24	<8	<8	<8

Remarks.

TA=Tube agglutination. HI=Hemagglutination inhibition. Chall.=Challenge. Immun.=Immunization.

Contag.=Contagion.

* Infection was performed in both immunized and untreated control groups 12 days after the final immunization. A group of chickens was placed in a cage housing containing 10 chickens which had been infected with aerosol of *M. gallisepticum* 2.3×10^5 CFU. All the chickens were sacrificed 4 weeks later.

Table 4. Relationship between doses and the number of times of aerosol immunization

Immunization		Challenge dose	2 weeks after challenge		4 weeks after challenge	
Antigen	Number of times (at 10 days' intervals)		Number protected/Total	Chickens protected	Number protected/Total	Chickens protected
5.0×10^9	3	2.0×10^4	8/10	80%	6/9	67%
1.0×10^9			6/10	60	6/10	60
2.0×10^8			2/10	20	3/9	33
5.0×10^9	2		6/10	60	5/9	56
1.0×10^9			3/10	30	3/9	33
2.0×10^8			2/10	20	1/10	10
None			3/10	30	0/10	0

Table 5. Protectivity of chickens maintained for various periods of time after immunization in respiratory tract with inactivated *M. gallisepticum* vaccine

Immunization at 10 days' intervals	Time after final immunization								
	1 month			3 months			6 months		
	Number protected/ Total		Mean CFU/Affected chickens*	Number protected/ Total		Mean CFU/Affected chickens	Number protected/ Total		Mean CFU/Affected chickens
5.0×10 ⁹ , 3 doses	8/10	80%	0.8	7/10	70%	48	3/9	33%	870
1.0×10 ⁹ , 3 doses	8/10	80	2.5	8/10	80	37	0/9	0	1700
5.0×10 ⁹ , 2 doses	4/10	40	4.0	2/10	20	180	1/10	10	1800
None	0/10	0	200	1/8	12	1500	0/10	0	3900

Remarks.

* Represented by mean of colony-forming units of *M. gallisepticum* isolated from organs of affected chickens in the respective test group.

were obtained from the group immunized intranasally with the vaccine as from that immunized intratracheally. The group immunized by spraying the vaccine, however, showed only a low degree of cell infiltration in the edematous wall of the air sac (Fig. 5).

Test of immune effect against contagious infection in chickens immunized with the vaccine aerosol: The results of the test are summarized in Table 3. Two groups immunized 2 and 3 times, respectively, showed no macroscopical lesions or positive bacterial cultivation. Of 9 non-immunized chickens, three were found to be infected contagiously. TA antibody titer increased only in the control group after the contagion.

Relationship between aerosol dosage and

protection: The results of examination are shown in Table 4. Three groups immunized with 3 doses of 5.0×10^9 and 1.0×10^9 CFU per chicken and 2 doses of 5.0×10^9 CFU per chicken showed a significant difference ($p \leq 0.2$) from any other group. With these effective immunizing doses, the following experiment was performed to examine the duration of immunity.

Persistence of immune state in aerosol-sprayed chickens: The results of this experiment are given in Table 5. They revealed that immune effect was significantly maintained in the 2 three-dose groups for at least 3 months. The mean number of isolated organisms was small in all the 3 groups, but the degree of suppression was low in the two-dose group.

Discussion

M. gallisepticum produces chronic respiratory disease in chickens. Its infection is highly contagious and known to lower body weight gain, egg production, and fertilization, and increase the number of waste chickens.

In order to protect chickens from this disease, the previous investigators administered live vaccine into the respiratory tract of birds, while they injected inactivated vaccine intramuscularly, intravenously, or subcutaneously.

In this study, it was found that the killed vaccine did not provide a significant protection when injected by the intramuscular route, regardless of the type of vaccine, but that plain vaccine provided protection when applied by the method of aerosol immunization in the respiratory tract. The results thus obtained suggest that immunity with killed organisms may induce a significant protectivity against the natural and experimental infection of *M. gallisepticum* only when the vaccine is introduced in the lower respiratory tract of chicken. The histopathological findings obtained lend support to the effect to vaccination. Intranasal instillation of the vaccine may not let the organism enter the deep tissue of the respiratory tract, while intratracheal inoculation may drive the organism downward.

At present, it is not known whether the immune mechanism given by plain inactivated vaccine is ascribed to antibodies formed or some other factor(s) or both. Antibody titration was negative in the serum after the completion of immunization even in the bird groups which later showed effective protection against challenge with the virulent organism. These findings suggest that the analysis of local

and cellular immunity may be necessary for interpretation of the results of observation obtained in the present investigation.

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Explanation of Figures

All the histological sections were stained with hematoxylin and eosin.

Fig. 1. Normal air sac of 56-day-old chicken.

Fig. 2. Air sac of non-immunized chicken 14 days after challenge. Remarkable cell infiltration and lymph follicle hyperplasia are recognized in the thickened wall of the air sac.

Fig. 3. Air sac of chicken injected intramuscularly with a mixture of inactivated *H. gallinarum* adsorbed to aluminum hydroxide and killed bacterial suspension, and sacrificed 14 days after challenge. Lymph follicle hyperplasia and cell

infiltration are seen in the wall of the air sac.

Fig. 4. Air sac of chicken injected directly with killed organisms of *M. gallisepticum* by the intratracheal route and sacrificed 14 days after challenge. Lymph follicle formation and cell infiltration are seen in the wall of the air sac.

Fig. 5. Air sac of chicken immunized with aerosol of killed organisms of *M. gallisepticum* and sacrificed 14 days after challenge. Only slight cell infiltration is found in the edematous wall of the air sac.