A Warm Hemagglutinin Induced in Chickens by Inoculation with *Mycoplasma gallisepticum** (33584)

H. E. ADLER AND A. J. DAMASSA

Department of Epidemiology and Preventive Medicine, University of California, Davis, California 95616

It has been known for a number of years that infection with *Mycoplasma pneumoniae* (MP) results in the development of specific antibodies and a cold hemagglutinin (7, 9). Although these cold hemagglutinins develop as a consequence of MP infection they were shown to be unrelated to the antibodies produced by the infection (6).

A similar situation exists in infections with avian mycoplasmas. Before *M. gallisepticum* (MG) was known to be the cause of infectious sinusitis of turkeys, Prier and Dart (8) demonstrated cold hemagglutinins (HA) in the sera of turkeys infected with sinus exudate from birds showing clinical signs of infectious sinusitis. Their results were erratic, however, and were negative in many instances where the tests were done at 25° or higher. Specific HA were produced only with red blood cells (RBC) of chicken origin, while those of sheep, swine, rabbit, and guinea pigs were agglutinated by normal sera. Prier and Dart further reported that serum from turkeys infected with *Salmonella* species and *Erysipelothrix insidiosa* also developed HA for chicken RBC at 5°.

In our laboratory it was noted that sera from chickens inoculated with MG occasionally developed HA for chicken RBC. This HA resists inactivation by 30 min at 56°, and its activity was routinely demonstrated at 37° and room temperature. To our knowledge this is the first report of this factor associated with mycoplasmal infections. This communication describes the warm HA and the factors which influence its development.

Cultures and antigens. Live organisms used in this study were propagated for 72 hr in Difco PPLO broth (without crystal violet) supplemented with 1% yeast autolysate (Albimi Laboratores) and 10% chicken or horse serum. The pH of the medium was 7.5. The fifth to the eleventh *in vitro* passages, containing 10^8 to 10^{10} viable organisms, were used.

Used in addition to live organisms were killed preparations of standard plate MG antigens prepared as described previously (1). *Salmonella* "O" antigen was prepared from a nonmotile strain of *S. typhimurium* by a standard procedure (3). *Pasteurella multocida* was prepared in a similar manner, and *Haemophilus gallinarum* antigen was obtained from Dr. R. Yamamoto of this Department. Some groups of birds were inoculated with equine serum or media as defined above but without mycoplasma.

Experimental chickens. Two breeds of birds were used. White leghorn chickens were from a closed flock obtained from the Poultry Husbandry Department of the University (Berkeley campus), while New Hampshire stock was supplied by Dr. W. W. Sadler of this Department. These fowls were free of mycoplasmas and other known viral or bacterial diseases. Neither parent flock is given vaccination of any kind. The birds used were of both sexes and 1.5–3.5 months old.

Origin and preparation of RBC. Erythrocytes were obtained from 7 chickens (4 New Hampshire and 3 white leghorns). The cells were collected by venous or cardiac puncture and placed immediately in tubes containing sodium citrate as anticoagulant. They were washed 3 times in physiological saline, harvested by centrifugation at 1500 rpm (270*g*), and then reconstituted in physiological saline at a concentration of 0.5%.

Bird inoculation. All birds received 1.0 ml for the intravenous inoculations unless otherwise noted. Intranasal and intratracheal immunization was accomplished by instillation

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TABLE I. Warm Hemagglutination Using Erythrocytes from White Leghorn or New Hampshire Donors with Sera from White Leghorn Chickens Infected Intravenously with *M. gallisepticum*.

Infected bird	Red blood cell donors						
	NH[a]1465	NH 1469	NH 1473	NH 7501	WL[a]444	WL 445	WL 446
301	U[b]	—	—	—	—	—	—
302	U	—	U	U	—	—	—
303	U	—	U	U	—	—	—
304	10[c]	—	10	10	—	—	—
307	U	—	10	10	—	—	—
308	U	—	U	—	—	—	—
309	10	—	20	10	—	—	—
393	—	—	U	—	—	—	—
394	U	—	U	U	—	—	—
395	U	—	20	10	—	—	—
397	U	—	40	10	—	—	—
396	U	10	U	—	—	—	—
400	—	—	U	—	—	—	—
390	U	U	U	—	—	—	—
305	U	10	U	—	—	—	—

[a] NH = New Hampshire; WL = white leghorn.
[b] U = undiluted.
[c] Reciprocal of the serum dilution.

of 2–3 drops of the live organisms into the nares or trachea of each bird.

Agglutination titers for MG. Rapid serum plate (RSP) titers were determined by procedures described previously (2).

Demonstration of hemagglutinins. Cold HA were assessed with doubling dilutions of serum in 13 × 100-mm clear glass tubes. Each tube contained 0.5 ml of serum dilution and 0.5 ml of RBC suspension. The reactants were placed in a refrigerator at 5° for 2 hr. The HA end point was the last tube (highest antiserum dilution) having complete hemagglutination of the RBC, as evidenced by a uniform deposit of the RBC over the entire curved surface of the test tube.

For assessment of warm hemagglutinins, one drop (approximately 0.025 ml) from a Pasteur pipette of doubling dilutions of the test serum was placed on a glass plate. To each drop was added an equivalent amount of RBC suspension, and the end point of HA at 10 min of rotation was recorded. Hemagglutination was recorded ranging from 4+ (complete HA) to 1+ (a barely visible reaction). The highest antiserum dilution representing a reaction intensity of 2+ or greater was taken as the hemagglutination end point. These warm HA titers were assessed at room temperature (22–25°) with both inactivated (56° for 30 min) and normal sera.

Fluorescent antibody studies. Fluorescein–isothiocyanate-labeled rabbit antichicken serum was conjugated by a described procedure (4) to determine the presence or absence of globulins attached to RBC.

Results. Trial 1. There was some question whether the source of RBC would influence HA results. Accordingly, 15 white leghorn chickens were inoculated intravenously with live MG and warm HA assessed with RBC from 4 New Hampshire and 3 leghorn chickens (Table I). One donor's (no. 1473) RBC appeared to be most reactive to the HA. Generally, HA activity was demonstrated to a greater degree whenever RBC of the opposite breed were used, i.e., New Hampshire RBC vs white leghorn sera.

Trial 2. Sixteen of 36 New Hampshire chickens inoculated intravenously with 1.0 ml of a standard MG plate antigen developed warm HA within 5 days. The titers ranged

TABLE II. *M. gallisepticum* Geometric Mean Titers and 95% Confidence Limits of Hemagglutination and Agglutination Titers of Sera from Vaccinated and Nonvaccinated Chickens Following Intravenous Challenge.

Days after challenge	RBC used[b]	Vaccinated challenge[f]			Unvaccinated challenge		
		Geometric mean[a]	Confidence limits		Geometric mean	Confidence limits	
			Lower	Upper		Lower	Upper
Hemagglutination titers							
6	1473	1.531[c]	1.072	2.185	10.54	7.121	15.60
	7501	1.84	0.980	1.429	3.903	2.403	6.337
13	1473	N[d]	N	N	1.429	1.268	1.611
	7501	N	N	N	1.342	1.192	1.511
Agglutination titers							
6[e]	—	259	179	373	1307	1112	1537
13	—	142	88	230	841	680	1040

[a] A negative titer was arbitrarily assigned a value of 1; undiluted = 2; 1:10 = 10, etc.
[b] All RBC were used as a 0.5% suspension and obtained from New Hampshire chickens.
[c] Reciprocal of the serum dilution.
[d] Negative.
[e] Rapid serum plate titers with *M. gallisepticum* antigen.
[f] Thirty-two birds were vaccinated and 33 unvaccinated.

between 1:2 and 1:32. During the same period, cold HA ranging in titer from 1:8 to 1:128 developed in 27 of 30 birds. Eight days after administration of antigen, all warm HA were absent but 8 of 30 birds still had cold HA titers ranging from 1:4 to 1:8. Heat treatment of the serum (56° for 30 min) did not alter the warm-HA titer. In addition, warm-HA titers did not differ between fresh and aged (4–5 days) RBC. Intravenous inoculation of sterile culture medium or equine serum failed to stimulate HA activity in 10 birds. Results were similar with sera from birds inoculated with *Salmonella, Pasteurella,* or *Haemophilus* antigens.

Trial 3. Twenty-four of 30 white leghorn chickens inoculated intravenously with 1.0 ml of live virulent MG developed warm HA. The titers ranged from 1:2 to 1:20.

Trial 4. To determine whether agglutination and HA coincided, 19 white leghorn and 38 New Hampshire chickens were inoculated intravenously with live organisms. In both breeds, warm HA appeared in 3 days, reached a peak in 7 days, and were absent in all birds 28 days postinoculation. Agglutinins to MG persisted.

Trial 5. A study was made to determine whether the antiglobulin test (Coomb's type reaction) would enhance the warm-HA reaction. No enhancement was noted. In MP infection, antibody is attached to RBC. We were unable to demonstrate attachment of globulins to RBC's from birds with HA-positive sera using fluorescein-labeled anti-chicken serum.

Trial 6. Warm HA developed in 2 of 10 New Hampshire chickens inoculated intranasally with live MG, and in 1 of 9 inoculated intratracheally. Upon intravenous challenge with live MG, only 1 bird responded with an HA titer whereas 8 of 10 previously normal birds developed HA.

Trial 7. RSP and HA titers of sera from 65 white leghorn chickens were evaluated statistically. Thirty-two of the birds were vaccinated intranasally with a drop of live *M. gallisepticum.* Three weeks after immunization, both groups of chickens were challenged intravenously with 1.0 ml having ap-

proximately 10^9 viable organisms/ml. The serum was tested for HA against the RBC of 2 donors (Table II). The HA and agglutination titers were significantly lower in the vaccinated group following challenge.

Trial 8. Specificity of HA as related to MG, when evaluated by cross absorption, revealed that HA were removed from the antisera by single absorption with MG, with a slight reduction in the RSP titer for MG. Absorption of HA-positive serum with RBC eliminated HA but had essentially no influence on the RSP titer.

Check for normal HA. A study of preinoculation sera in this and other investigations revealed 1 positive HA serum at the undiluted level in 350 normal chickens tested.

Discussion. A warm HA against chicken RBC commonly develops after intravenous inoculation of large numbers of viable cells of MG. Hemagglutinins are induced also by inoculating large numbers of killed MG, though with lower frequency. There was little, if any relationship between agglutinating RSP MG titers and HA. Liu *et al.* (6) also demonstrated with cross absorption that the antibody measured by the fluorescent antibody technique was unrelated to the HA induced by MP infection. Unlike in findings (5) with human patients infected with MP, we have not been able to demonstrate attached globulins to RBC from affected chickens by the antiglobulin (Coomb's) procedure.

Summary. Warm hemagglutinins were produced in chickens inoculated with suspensions of living or dead *Mycoplasma gallisepticum*, with higher titers being produced by living organisms. Killed suspensions of *Salmonella typhimurium, Pasteurella multocida* or *Haemophilus gallinarum* gave negative results. The peak of *M. gallisepticum* rapid serum plate agglutination did not appear to be correlated with hemagglutination titers.

1. Adler, H. E. and DaMassa, A. J., Appl. Microbiol. **15**, 245 (1967).

2. Adler, H. E. and DaMassa, A. J., Appl. Microbiol. **16**, 558 (1968).

3. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., "Methods in Immunology," p. 76. Benjamin, New York (1963).

4. Corstvet, R. E. and Sadler, W. W., Poultry Sci. **43**, 1280 (1964).

5. Feizi, T., Ann. N. Y. Acad. Sci. **143**, 801 (1967).

6. Liu, C., Eaton, M. D., and Heyl, J. T., J. Exptl. Med. **109**, 545 (1959).

7. Peterson, O. L., Ham, T. H., and Finland, M., Science **97**, 167 (1943).

8. Prier, J. E. and Dart, G. Cornell Vet. **41**, 38 (1951).

9. Turner, J. C., Nature **151**, 419 (1943).

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