

Studies on Hemagglutination by *Chlamydia** (34044)

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The agglutination of fowl erythrocytes (RBC) by psittacosis agent has been the subject of a previous communication (1). In this report it was noted that allantoic fluids infected by psittacosis agent, in addition to agglutinating mouse RBC, has the capacity to agglutinate RBC from certain chickens and that this selectivity correlated with the hemagglutinating activity of vaccinia virus preparations. The present report represents a continuation of this work and includes information on hemagglutination by other members of the *Chlamydia* group.

Materials and Methods. *Chlamydia*: Psittacosis (6BC) was obtained from Dr. H. Morgan, University of Rochester and also from the American Type Culture Collection (ATCC); meningopneumonitis, MP, (Francis); feline pneumonitis, FP, (No. 1); mouse pneumonitis, MoP, (Nigg II) were all obtained from ATCC.

Viruses. The strain of vaccinia virus used in this study was originally derived from commercial calf lymph vaccine. The virus has been passaged several times on the chorio-allantoic membrane of the chick embryo and in primary rhesus monkey kidney cell cultures.

Hemagglutinin. Chlamydial agents were passaged several times in the yolk sac of the chick embryo; psittacosis and MP were readily passaged in the allantoic cavity. Allantoic fluids rich in elementary bodies were used as seed for production of hemagglutinin. Eight to 9-day-old embryos were inoculated with 0.2 ml each of stock material diluted in brain heart infusion broth (Difco) containing

4000 μ g of streptomycin/ml. Best results were obtained with FP and MoP when 8-day-old embryos were inoculated with 0.5 ml of a 20–50% yolk sac suspension. Embryos were inoculated each time with yolk sac suspension.

Allantoic fluids were collected from infected embryos, pooled, and tested for hemagglutinating activity. Hemagglutinin was separated from the elementary bodies by centrifugation at 10,000 rpm in a Sorvall (RC-2) centrifuge. Fluids from FP-and MoP-infected embryos were not centrifuged since hemagglutinating activity was usually low with these agents and only infrequently encountered. Vaccinia hemagglutinin was prepared by collecting material from infected cell cultures and was used without further purification.

Hemagglutination test. Mouse blood was collected fresh each time in ACD (Acid Citrate Dextrose B, Baxter Lab.). Human and dog blood were also collected in ACD. Sheep blood was collected in Alsever's solution. Avian blood was collected in 4.8% sodium citrate. Roosters were obtained from a local commercial poultry source and the two breeds tested were White Leghorn (WL) and New Hampshire Red (NHR). RBC were washed with diluent consisting of one part of McIlvaine buffer and four parts 0.15 M saline. This diluent was used throughout the study. The hemagglutination test was performed in tubes and the endpoint was taken as the highest dilution which gave a definitely positive pattern of hemagglutination. In this report the designation chicken⁺ will be used for RBC collected from WL birds which were agglutinated by psittacosis and vaccinia, and chicken⁻ will be used for RBC collected from NHR which were not agglutinated.

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Hemagglutination-inhibition (HI) test. Chicken sera inactivated at 56° for 30 min were preabsorbed with mouse RBC and the procedure followed was essentially that described by Hilleman *et al.* (2). Four units of HA were used. The HI endpoint was the highest dilution of serum which gave complete inhibition of hemagglutination. Positive control sera were prepared by immunizing a WL chicken and a rabbit with allantoic fluid infected by psittacosis agent and pretreated with ultraviolet light to inactivate infectivity.

Absorption of hemagglutinin. Psittacosis hemagglutinin was absorbed as follows: 40 ml of 1:4 dilution of hemagglutinin was added to 0.8 ml of packed mouse RBC or chicken⁺ RBC. The mixture was allowed to react for 2 hr at room temperature on a mechanical shaker. The RBC were removed by centrifugation and an aliquot of the absorbed allantoic fluid was immediately tested for hemagglutinating activity. The absorption procedure was repeated as many times as required.

Agglutination using slides. A volume of 0.1-ml 0.05% chicken⁺ RBC suspension was added to 0.2 ml of hemagglutinin contained in a tube. A small drop of mixture was then placed on an 18 mm² coverslip and incubated for 60 min at room temperature in a petri dish containing moistened filter paper. After incubation, a drop of 0.1% mouse RBC suspension was carefully placed over the first drop. The drops were gently mixed using a fine glass rod and the coverslip was covered with a microconvexity slide and the slide reverted immediately. After further incubation for 1 hr the slides were examined microscopically.

Results. Former work in Israel had shown (1) that RBC from White Leghorn chickens were susceptible to agglutination by psittacosis agent and vaccinia virus whereas, RBC from "Rhode Island" Red chickens were generally insusceptible. Additional evidence for this phenomenon was sought in chickens from the area of Buffalo, New York. Blood was collected from local WL and NHR birds and their RBC tested for hemagglutination by psittacosis agent and vaccinia virus. A high incidence of positive reactions was found for

TABLE I. Agglutination of Chicken RBC by Various *Chlamydia*.

<i>Chlamydia</i>	RBC		
	Chicken +	—	Mouse
Psittacosis	128 ^a	<2	64
Meningopneumonitis	16	<2	16
Feline pneumonitis	16	<2	16
Mouse pneumonitis	16	<2	8
Vaccinia	64	<2	<2

^a Hemagglutination titer.

RBC from WL (94.7%) and a low incidence for NHR (10.0%). RBC which were agglutinated by psittacosis agent were always agglutinated by vaccinia.

Experiments were undertaken to investigate other members of the *Chlamydia* group of agents which have been reported to have hemagglutinating activity (2-4). The results have been summarized in Table I. Infected allantoic fluids obtained from MP, FP, and MoP agents were found to have the capacity to agglutinate chicken RBC as well as mouse RBC. Chicken⁻ RBC were not agglutinated by any of the above agents.

It has been reported by Datt (5) that turkey RBC were agglutinated by vaccinia. Accordingly, turkey RBC were tested in this study against vaccinia, psittacosis, and MP preparations. Positive results were obtained in each case. In order to establish that the hemagglutinins studied had no unusual agglutinating properties, psittacosis, MP, and FP were also tested against dog, sheep, human, and peacock RBC with negative results.

It was of interest to determine if chickens with sensitive RBC had any previous serologic experience with psittacosis (ornithosis) agent. Sera collected from WL and NHR birds were examined for antibodies in the HI test. Rabbit and chicken antisera against psittacosis and a pool of normal chicken serum obtained from commercial sources for cell culture use were also tested. No difference was noted in the HI antibody titers between the WL and NHR chickens. Titers ranged from <4 to 16; the titer obtained with commercial chicken serum pool was 16.

TABLE II. Absorption of Psittacosis Hemagglutinin.

	RBC	
	Chicken ⁺	Mouse
Unabsorbed	64 ^a	64
Absorbed with chicken ⁺ RBC		
1X	16	32
2X	<4	8
3X	<4	<4
Absorbed with mouse RBC		
1X	<4	8
2X	<4	<4

^a Hemagglutination titer.

A series of experiments was carried out to determine if there were two hemagglutinins present in psittacosis infected allantoic fluids, one specific for mouse RBC and the other for chicken RBC. Absorption with either mouse or chicken⁺ RBC resulted in removal of hemagglutinating activity for both chicken⁺ and mouse RBC (Table II). When psittacosis hemagglutinin was absorbed once with a relatively large volume of mouse or chicken⁺ RBC, removal of hemagglutinating activity for RBC of both species was also found. Similar results were obtained when MP hemagglutinin was absorbed. Absorption of hemagglutinin with chicken⁻RBC or sheep RBC failed to remove hemagglutinating activity. In these experiments absorptions were performed using hemagglutinins diluted 1:4. Undiluted hemagglutinin usually fails to form patterns of agglutination but dialyzed hemagglutinin yields positive results (2). Absorption of dialyzed undiluted psittacosis hemagglutinin with either mouse or chicken⁺ RBC also resulted in removal of activity. These data suggested that only one hemagglutinin was present in the preparations.

One might expect that chicken⁺ and mouse RBC would form patterns of mixed agglutination if they were reacted with hemagglutinin under appropriate conditions. Experiments were performed using slides following several procedures. Best results were obtained with the one described in *Materials and Methods*. When psittacosis hemagglutinin was allowed to react with chicken⁺ and

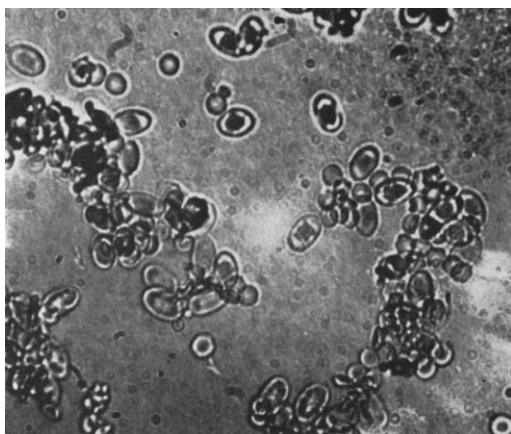


FIG. 1. Mixed agglutination of chicken⁺ and mouse RBC by psittacosis agent. Photographed using 10X ocular and 43X objective.

mouse RBC, mixed agglutination patterns were readily observed (Fig. 1). Best results with psittacosis hemagglutinin were obtained at room temperature. However, with vaccinia the strongest patterns of agglutination were formed when the slides were incubated at 36°. Only chicken⁺ RBC were agglutinated by vaccinia with mouse RBC free in the preparation. These experiments would also support the concept of the presence of one hemagglutinin in the chlamydial preparations.

Discussion. *Chlamydia* hemagglutinins may be added to the list of substances, including vaccinia hemagglutinin, Coxsackievirus A7 hemagglutinin, extracts of human RBC, fowl and human tissues, and Kahn antigen, which possess the property of agglutinating RBC from selected chickens. Recently, Zakay-Rones *et al.* (6) reported that sonicates of purified elementary bodies of trachoma agent selectively agglutinated chicken RBC.

The difference in sensitivity to psittacosis and vaccinia hemagglutinins of RBC from White Leghorn and New Hampshire Red chickens in the present study was compatible with data presented earlier (1). Evidence for higher sensitivity of RBC from leghorns to vaccinia hemagglutinin as compared to other breeds has been reported (7). However, not all investigators have found these differences. Youngner and Rubinstein (8) noted

that only 15% of White Leghorn chickens tested by them had RBC which were agglutinable by vaccinia virus. Williamson (9) on the other hand, found that only 6% of RBC of Rhode Island Red chickens were insensitive to vaccinia and Coxsackievirus A7 hemagglutinins. Recently, Gilmour and Allison (11) have presented additional data for the genetic basis of susceptibility of chicken RBC to hemagglutination by vaccinia and Wasserman antigen.

The results obtained in our studies with other *Chlamydia* agents (meningopneumonitis, feline pneumonitis, mouse pneumonitis) demonstrate that the capacity to agglutinate chicken RBC is not restricted to psittacosis agent in the group. The data would indicate that only one hemagglutinin is present in the chlamydial preparations which is capable of agglutinating both mouse and chicken RBC. O'Connell and Milgrom (10) found two hemagglutinins with different specificities for chicken and human RBC in their studies on Newcastle disease virus.

Summary. A high incidence of susceptibility to psittacosis and vaccinia hemagglutinins was found for erythrocytes from White Leghorn whereas a low incidence was found in New Hampshire Red chickens. Meningopneu-

monitis, feline pneumonitis, and mouse pneumonitis agents also selectively agglutinated chicken erythrocytes. Evidence was obtained for the presence of only one hemagglutinin in chlamydial preparations capable of agglutinating chicken and mouse erythrocytes.

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