Hemagglutination of Chicken Erythrocytes by the Agent of Psittacosis.* (30186)

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The phenomenon of hemagglutination has been described for certain members of the psittacosis-lymphogranulomavenereum-trachoma (PLT) group of agents(1,2,3). One interesting aspect has been the restriction of hemagglutination to only mouse erythrocytes (rbc). In the present study the species range is extended in that it was found that psittacosis agent was also capable of agglutinating chicken rbc. Hemagglutination of chicken rbc was found to be confined only to cells susceptible to agglutination by vaccinia.

A description of this phenomenon is the subject of this report.

Materials and methods. Viruses. Psittacosis (6BC strain) was originally obtained from Miss L. Hanna, San Francisco, and was propagated in this laboratory in FL cell cultures in LY medium consisting of 80% Earle's solution containing 0.45% glucose, 0.1% yeast extract (Difco), 0.5% lactalbumin hydrolysate (N.B.C.), 20% inactivated human serum plus 0.001% phenol red and 200 μg/ml streptomycin. Noguchi strain of vaccinia was maintained in this laboratory by passage on the chorioallantoic membrane (CAM) of the chick embryo(4). The strain was also propagated in a line of continuous human kidney cells maintained in LY medium, substituting 5% calf serum for human serum.

Antisera. The following hyperimmune rabbit antisera were used: (a) Anti-psittacosis (6BC), immunized with arcton purified yolk sac antigen, (b) Anti-trachoma (i) strain Camal(5), immunized with concentrated yolk sac antigen (ii) strain T'ang, immunized with FL cell culture-grown antigen, (c) Anti-normal chick embryo yolk sac, immunized with concentrated yolk sac antigen, (d) Anti-vaccinia, immunized with 20% suspension of CAM grown virus, (e) Normal rabbit serum. Preparation of antisera (a)(b)(c) is described in greater detail elsewhere(6).

Hemagglutinin. Psittacosis was passaged one to two times in the allantoic cavity of the chick embryo and allantoic fluid, rich in psittacosis agent, served as a stock for subsequent hemagglutinin production. Eight- to nine-day-old chick embryos were inoculated with 0.2 ml of stock material containing 4,000 μg streptomycin/ml. When 20-30% of the embryos were dead, usually after 4 days incubation at 35°C, the remaining living embryos were refrigerated overnight and the allantoic fluid collected next day. Each fluid was tested and only those with a hemagglutinin titer of 1:32 or higher were accepted. Hemagglutinin was prepared essentially by the method of Gogolak and Ross(3). Allantoic fluids were pooled and centrifuged at 1,000 rpm for 10 minutes at 5°C and then recentrifuged at 8,500 rpm (Servall) for 35 minutes at 5°C. The clear supernatant was distributed in small aliquots and stored at —60°C. Vaccinia preparations were either 20% suspensions of CAM or cell culture fluids which were clarified by centrifugation after sonication.

Hemagglutination test. Blood was collected in Alsever's solution and the cells washed with a diluent consisting of one part of McIlvaine buffer (pH 6.9-7.0) and 4 parts of 0.85% saline. Unless indicated otherwise, this diluent was used throughout the study. Mouse blood was collected fresh each time and chicken blood was stored in the refrigerator as a 10% suspension for 3-4 days or as whole blood for longer periods. The hemagglutination test for psittacosis followed the procedure of Hilleman *et al*(1). A volume of 0.3 ml washed rbc suspension was added to each tube (0.5 ml) in a series of 2-fold

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TABLE I. Comparison of Hemagglutination of Chicken and Mouse Erythrocytes by Vaccinia and Psittacosis.

		Hemagglutination titer					
		Chicken 1		Chicken 2		Mouse	
Erythrocyte conc		.5%	1.0%	.5%	1.0%	.5%	1.0%
Vaccinia	CAM	32*	32	0	0	0	0
	Cell culture	32	32	0	0	0	0
Psittacosis (hemagglutinin)	Exp 1	512	128	0	0	128	64
	" 2	1024	128	0	0	128	64

* Reciprocal of hemagglutination endpoint.

dilutions of hemagglutinin. The endpoint was taken as the highest dilution of material which gave complete hemagglutination. One unit of hemagglutinin was considered to be contained in 0.5 ml of the endpoint dilution. Vaccinia hemagglutination was performed by the procedure used for psittacosis.

Hemagglutination inhibition test. Antisera used in tests with chicken rbc were pre-absorbed with chicken cells and antisera in tests with mouse rbc were pre-absorbed with mouse cells. Absorption was based on the method of Matumoto *et al*(7). A 1:2 dilution of serum, heat inactivated at 56°C for 30 minutes, was mixed with an equal volume of a 10% suspension of erythrocytes. For chicken cells the mixtures were incubated for 1 hour at 37°C prior to absorption in the refrigerator overnight, with gentle shaking. The mixtures were then centrifuged at 1,500 rpm for 10 minutes and the clear supernatant removed. This was considered as 1:4 dilution of the serum. The hemagglutination inhibition test for psittacosis was based on the procedure of Hilleman *et al*(1) using 4 hemagglutinating units in a volume of 0.25 ml. For vaccinia the hemagglutination inhibition test as described by Kempe(8) was employed using 2 hemagglutinating units per 0.25 ml.

Results. In addition to the hemagglutination of mouse rbc, agglutination of chicken erythrocytes which were susceptible to agglutination by vaccinia was also obtained (Table I). Hemagglutination titers were usually higher with chicken than with mouse rbc. A concentration of 0.5% for chicken rbc and 1.0% for mouse was considered optimum and was used throughout the study. Chicken cells settled faster and the test could usually be read after 40-60 minutes while tests with mouse cells usually required 60-90 minutes. When untreated psittacosis-infected allantoic fluid was compared to hemagglutinin prepared by centrifugation, as described in *Materials and methods,* similar hemagglutination titers were obtained with either chicken or mouse rbc's. Preparations of hemagglutinin were used for the remainder of the study.

Blood was collected from additional chickens and their rbc's tested for sensitivity to vaccinia and psittacosis hemagglutinins (Table II). Erythrocytes sensitive to vaccinia were found to be sensitive to psittacosis hemagglutinin. Vaccinia negative rbc were also negative with psittacosis. It is interesting that chicken 1 (Table I) and chickens 3-10 (Table II) were purebred, White Leghorns (8/9 pos., 88%). Chickens 11-15 (Table II) were Rhode Island Reds (1/5 pos., 20%). Chicken 2 (Table I) whose cells were negative for vaccinia and psittacosis was a crossbreed of unknown identity.

Hemagglutination of chicken and mouse rbc was tested in different diluents with psittacosis hemagglutinin as summarized in Ta-

TABLE II. Sensitivity of Erythrocytes from Different Chickens to Vaccinia and Psittacosis Hemagglutinins.

	Hemagglutination titer			Hemagglutination titer	
Chicken No.	Vaccinia	Psittacosis	Chicken No.	Vaccinia	Psittacosis
3	32*	1024	10	<2	<2
4	64	512	11	<2	<2
5	32	32	12	<2	<2
6	32	32	13	<2	<2
7	16	64	14	<2	<2
8	32	64	15	512	256
9	16	64			

* Reciprocal of hemagglutination endpoint.

TABLE III. Comparison of Different Diluents for Hemagglutination of Chicken and Mouse Erythrocytes by Psittacosis Hemagglutinin.

	Erythrocytes	Dilution of hemagglutinin 1: 0	2	4	8	16	32	64	128	256	512	1024	2048	4096
McIlvaine buffer 1 part + 4 parts saline .85%, pH 7.0	Chicken	0	++	++	++	++	++	+±	+	+	+	±	0	0
	Mouse	0							0	0	0	ND	ND	ND
Saline .85%, pH 7.0	Chicken	0	0	++	++	++	++	+±	+	+	+	+	±	0
	Mouse	0	0	±					0	0	0	ND	ND	ND
Saline .85% + .002M phosphate, pH 7.0	Chicken	0	0	++	++	++	+	+	+	±	±	±	±	0
	Mouse	0	0				0	0	0	0	0	ND	ND	ND
Saline .85% + magnesium sulfate .1%, pH 6.8	Chicken	ND	0	0	0	0	0	0	0	0	0	0	0	0
	Mouse	ND	0	0	0	0	0	0	0	0	0	ND	ND	ND

0 = Negative. + = Complete hemagglutination. ± = Partial hemagglutination. ND = Not done.

ble III. Inhibition was frequently found with either chicken or mouse rbc's when undiluted hemagglutinin was used. When plain saline or saline mildly buffered with phosphate was used as diluent, this inhibition was also found at 1:2 dilution and sometimes higher. Inhibition in low dilutions was not noted with vaccinia in any of the diluents tested. When 0.1% magnesium sulfate was incorporated in plain saline, hemagglutination was inhibited for both chicken and mouse erythrocytes. Vaccinia hemagglutinin, however, was not affected by the incorporation of magnesium sulfate. The agglutination of chicken and mouse erythrocytes was also tested at 37°C, room temperature (26°C) and at 5°C (refrigerator) at pH 7.0. Similar results were obtained at 37°C and room temperature. In the cold, a zone of inhibition was noted followed by weak patterns of agglutination and generally the titers were lower than at 37°C or room temperature. Mouse cells did not react as well in the cold as chicken cells. Hemagglutination of chicken cells with vaccinia was equally good at the three temperatures tested.

Hemagglutination inhibition tests were performed following the procedures described in *Materials and methods*. Vaccinia hemagglutinin produced in the chorioallantoic membrane (CAM) and in cell culture was used. Both chicken and mouse rbc were used in the tests performed with psittacosis hemagglutinin. The results were tabulated in Table IV. No cross reaction between vaccinia and psittacosis in the hemagglutination in-

TABLE IV. Hemagglutination Inhibition Tests with Vaccinia and Psittacosis Hemagglutinins.

	Hemagglutination inhibition titer			
	Vaccinia		Psittacosis	
	Chicken		Chicken	Mouse
Antisera	CAM	Cell culture		
Anti-vaccinia	256	512	4	<4
Anti-psittacosis	<4	<4	128	32
Anti-trachoma				
Camal strain	<4	<4	512	128
T'ang "	<4	<4	64	32
Normal rabbit	<4	<4	16	<4
Anti-normal chick embryo yolk sac	<4	<4	8	4

hibition test was obtained. Similar results were obtained for vaccinia with either the CAM or cell culture preparations. Vaccinia also failed to react with the anti-trachoma sera. Chicken and mouse rbc gave similar results with psittacosis hemagglutinin although chicken cells appeared to be more sensitive not only to specific antibody, but also to inhibitors present in normal rabbit serum. Hemagglutination inhibition was obtained with psittacosis hemagglutinin and anti-trachoma sera. One anti-trachoma serum gave a higher titer than that obtained with the anti-psittacosis serum.

Discussion. Hemagglutination by members of the psittacosis group of agents has generally been considered to be restricted to mouse erythrocytes. Hilleman *et al*(1) reported that meningopneumonitis (ornithosis) virus failed to agglutinate pigeon, chicken, domestic duck, ring neck pheasant, quail, starling, hamster, guinea pig, sheep and human O cells. Horse, cattle, pig, sheep, goat, rabbit, guinea pig, chicken, pigeon and human O cells were reported as negative for psittacosis (avian strain Budgerigar No. 1) by Inaba *et al*(9). Since the report of Nagler(10) on hemagglutination by vaccinia, numerous reports have appeared in the literature attesting to the fact that only the cells of certain chickens can be agglutinated by vaccinia virus. In the present study it was found that erythrocytes from chickens which were sensitive to vaccinia hemagglutinin could also be agglutinated by psittacosis (6BC) hemagglutinin. Erythrocytes from vaccinia negative chickens were also negative for psittacosis. Failure to obtain hemagglutination with chicken cells in the past could be accounted for if the chicken cells tested were negative for vaccinia. The limited data obtained in the present study would lead to the speculation that the sensitivity of chicken cells to agglutination by vaccinia and psittacosis might be a genetic factor affected by the breeding history of the chickens. The data of Suzuki *et al*(11) would support this view.

One of the disadvantages of the hemagglutination test for the psittacosis group has been the lability of the mouse erythrocytes used in the test. For best results only fresh rbc should be used. Chicken cells have been found to store quite well as 10% suspensions, or longer as whole blood. Stored cells have been found to retain their sensitivity and this eliminates the requirement for fresh blood. At the present time, other hemagglutinin producing members of the psittacosis group, meningopneumonitis, feline pneumonitis and murine pneumonitis agents have not been tested for their ability to agglutinate chicken erythrocytes.

It was recognized early in the examination of hemagglutinins of the psittacosis group that a resemblance to poxvirus hemagglutinins existed(3). Several biologic differences between vaccinia and psittacosis hemagglutinins were noted during this study. In accordance with previously published data vaccinia virus failed to hemagglutinate mouse erythrocytes, whereas positive results were obtained with psittacosis. In this regard psittacosis behaves more like ectromelia in agglutinating both chicken and mouse cells (12). Magnesium sulfate in saline inhibited psittacosis hemagglutinin and weak reactions were obtained at 5°C. No inhibition of hemagglutination was noted for vaccinia in the presence of magnesium sulfate or in low dilutions in any diluent and similar reactions were obtained at 5°C as at 37°C or room temperature. The failure to obtain cross reactions in the hemagglutination inhibition test points to the fact that the hemagglutinins are specific for the respective agent, and that while vaccinia and psittacosis are distinct serologically, the hemagglutinating moiety of their hemagglutinins are similar in their capacity to react with receptors on erythrocytes of certain species.

The cross reactions obtained with psittacosis hemagglutinin and anti-trachoma sera would indicate that the hemagglutinin is group specific for the psittacosis-lymphogranulomavenereum-trachoma (PLT) group. Goto *et al*(13) in Japan, tested sera from a healthy group of school-children and a group with trachoma. A higher incidence of positive results and higher average titers were obtained with the trachoma group using either the complement fixation test or hemagglu-

tination inhibition test with psittacosis antigens.

Summary. Psittacosis agent was found to hemagglutinate chicken erythrocytes susceptible to agglutination by vaccinia virus. Negative results were obtained with vaccinia-negative cells. The agglutination of chicken cells by psittacosis was similar to mouse cells with reference to conditions for hemagglutination, sensitivity to inhibitors and the hemagglutination inhibition test. Differences between vaccinia and psittacosis hemagglutinins were found. No cross serologic relationship was found between psittacosis and vaccinia in the hemagglutination inhibition test. Positive hemagglutination inhibition tests were obtained with anti-trachoma sera using psittacosis hemagglutinin and either chicken or mouse cells.

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1. Hilleman, M. R., Haig, D. A., Helmold, R. J., J. Immunol., 1951, v66, 115.

2. Gogolak, F. M., J. Inf. Dis., 1954, v95, 220.

3. Gogolak, F. M., Ross, M. R., Virol., 1955, v1, 474.

4. Zakay-Roness, Z., Rosin, A., Bernkopf, H., J. Immunol., 1962, v88, 184.

5. Bernkopf, H., Treu, G., Maythar, B., Arch. Ophthal., 1964, v71, 693.

6. Katzenelson, E., Bernkopf, H., J. Immunol., 1965, v94, 467.

7. Matumoto, M., Goto, T., Nakamura, H., Matsushima, S., Naito, H., Shioda, H., Omori, T., Jap. J. Exp. Med., 1960, v30, 327.

8. Kempe, C. H., in Diag. Proc. for Virus and Ricketts. Dis., A.P.H.A., 1956, 341.

9. Inaba, Y., Omori, T., Ishii, S., Jap. J. Exp. Med., 1957, v27, 425.

10. Nagler, F. P., Med. J. Aust., 1942, v1, 281.

11. Suzuki, S., Fuwa, A., Fujii, R., Kurimoto, U., Zentr. Bakteriol. Parasitenk., 1955, v162, 405.

12. Burnet, F. M., Boake, W. C., J. Immunol., 1946, v53, 1.

13. Goto, T., Matumoto, M., Nakamura, H., Naito, H., Matsushima, S., Morita, S., Jap. J. Exp. Med., 1961, v31, 237.

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Effect of Actinomycin D on Morphine Tolerance.* (30187)

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There is indirect evidence suggesting that tolerance to morphine may be related to the production, in the central nervous system, of proteins or peptides capable of counteracting the effects of the drug(1-5). The purpose of the present study is to determine whether development of tolerance to the analgesic action of morphine can be prevented by drugs which inhibit protein synthesis.

Methods and materials. a) *Mice.* Tolerance was induced in Swiss albino mice (20 to 25 g, both sexes) obtained from Thomas E. Euers, Austin, Texas by the following schedule of subcutaneous injections of morphine sulfate: First day, 2 injections of 0.12 mg per mouse (4.8 to 6.0 mg/kg); second day, 3 injections of 0.22 mg per mouse (8.8 to 11 mg/kg) and third day, 2 injections of 0.28 mg per mouse (11.2 to 14 mg/kg). The interval between daily injections was 4 hours.

Response to noxious stimuli was tested by Haffner's method as modified by Bianchi and Franceschini(6). The mice were screened before the experiment and those which did not respond to the standard pressure applied to the base of the tail for 10 seconds were eliminated. The analgesic effect of 0.15 mg of morphine sulfate per mouse (6.0 to 7.5 mg/kg) was determined the day before starting the injection schedule to produce tolerance and on days 1, 3 and 4 during the establishment of tolerance. The results are expressed in terms of the per cent mice re-

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