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The Serological Activity of Extra-embryonic Fluids of Chick Infected with Virus of Influenza A.

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The use of the fertile hen's egg for the propagation and study of the virus of influenza A has been limited to the chorio-allantoic membrane, the embryo and the yolk sac. Nothing was known of the virus content of the allantoic and amniotic fluids until the recent publication of Burnet[1] whose findings agree with the observations made in this laboratory during the past several months.

Several mouse-adapted strains of influenza A virus have been transferred to chick embryos by injecting 0.5 ml amounts of mouse lung filtrates or pooled extra-embryonic fluids through the air space into the allantoic sac of fertile eggs which previously had been incubated for 9 to 10 days. Accidental injection into the amnion, embryo or yolk sac was unlikely because of the mobility of these structures. After further incubation for 24 to 48 hours the extra-embryonic fluids (amniotic and allantoic) were usually harvested together and were studied separately in only a few experiments. From 3 to 8 ml of fluid were obtained from the allantoic sac and 1.0 to 2.5 ml from the amnion. The amniotic fluid was frequently quite clear while the allantoic fluids showed various degrees of turbidity. On standing in the refrigerator, or on freezing and thawing, a precipitate was formed in many of the allantoic fluids, which was removed by centrifugation without apparent loss of serological activity.

The extra-embryonic fluids were both very rich in virus. For example, the WS-strain,[2] now in its 48th consecutive egg passage not only retained its virulence for mice but actually became lethal in much higher dilutions in these fluids than the original mouse lung material. Preparations were occasionally encountered of which as little as 0.05 ml of a 10^{-10} dilution proved fatal for white mice. The intraäbdominal injection of such fluids produced immunity in mice against the mouse-adapted influenza strains, and the anti-

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1 Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sc.*, 1940, **18**, 353.

2 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet*, 1933, **2**, 66.

influenzal mouse lung serum neutralized the egg-adapted virus. The F-12[3] and Fla-12[4] strains, in the 25th consecutive egg passage, behaved in a similar way. Other strains have been started too recently to allow inclusion in this report.

Since these virus preparations are obtained without grinding of tissues they seem to be particularly suited for studies in the purification of influenza virus as the interference of normal cellular components[5] is reduced considerably. The low content of non-viral proteins (total protein 0.03 to 0.05%) in this fluid in connection with its high virus titer suggests its suitability as a vaccine. When produced under conditions defined below the fluids, and the allantoic fluid in particular, contain a satisfactory titer of complement fixation antigen and show a flocculation phenomenon when mixed with convalescent or immune sera.

The application of the complement fixation reaction to influenza A is well established.[6] For this test dilutions of the pooled extra-embryonic fluids, serum and complement were mixed in equal volumes (0.2 ml) and incubated for one hour at 37°C. After the addition of 0.4 ml of sensitized sheep cells (2.5%) the test was further incubated for 60 minutes when final readings were made. Two units each of amboceptor and complement were employed. Anticomplementary reactions, so frequent with mouse lung antigens have not been observed with these fluids. Normal egg fluid never gave complement fixation in the presence of convalescent or immune sera.

In contrast with the complement fixation reaction little is known about aggregation phenomena with preparations containing influenza A virus. Magill and Francis[7] observed a flocculation of crude influenzal mouse lung suspensions by human convalescent sera. Our attempts to obtain a specific agglutination of influenza A virus preparations, derived from infected mouse lungs by high speed centrifugation, were unsuccessful. Whether this failure was due to insufficient amounts of virus[8] or to a mechanical hindrance through normal lung particles[5] present in these suspensions is unknown. Mouse lung preparations with as much as 10^8 to 10^{10} infectious units per ml for white mice did not clump in the presence of im-

[3] Stokes, J., Jr., and Wolman, I. J., *New International Clinics*, 1940, **1**, 115.

[4] Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

[5] Henle, W., and Chambers, L. A., *Science*, 1940, **92**, 313.

[6] Nigg, C., Crowley, L. K., and Wilson, D. E., *Science*, 1940, **91**, 603. (Earlier references given here).

[7] Magill, T. P., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 81.

[8] Merril, M. H., *J. Immunol.*, 1936, **30**, 169.

mune sera against tissue culture virus, or of convalescent sera. However, when the pooled extra-embryonic fluids, clarified by horizontal centrifugation, were mixed with equal amounts of human or ferret convalescent sera (0.2 ml each), a flocculation was observed after incubation for one hour at 37°C, overnight refrigeration, and centrifugation for 10 minutes at 1500 r.p.m. The test was read after gentle tapping of the tubes. The floccules rising from the bottom tended to redisperse when shaken too vigorously.

The complement fixation and flocculation antigens appear in the fluids simultaneously, but decidedly later than the infectivity. Table I shows that the pooled extra-embryonic fluids contained a high titer of virus after 12 hours while the complement fixation test was still negative. After 24 hours this reaction became slightly positive and reached its peak in 36 hours. Also the infectivity was highest at this time but decreased thereafter, while no such decrease was noted in the serological reactions and the flocculation, following otherwise closely the complement fixation reaction, was even more pronounced after 48 hours.

In order to obtain, therefore, sufficient complement fixation antigen, the allantoic sac should be inoculated with a dose of virus sufficiently dilute to allow the embryos to survive at least for 36 hours. The time of survival naturally depends on the dose of virus used as inoculum. Table II shows an experiment in which groups of 8 eggs were infected with different amounts of virus, and the fluids were harvested and pooled when 30 to 60% of the embryos had died. Thus the fluids of eggs inoculated with a more concentrated preparation (undiluted or 10^{-2}) had to be harvested after 24 hours, while the fluids of those injected with higher dilutions

TABLE I.

Test	Dilution of fluid	WS-42 (extra-embryonic fluids) harvested after 6 hr	12 hr	24 hr	36 hr	48 hr
Mouse Titration	10^{-5}	3 3 3	D6 D7 D10	D6 D6 D8	D5 D5 D6	D6 D7 D10
	10^{-6}	0 0 0	D8 D9 D10	D6 D11 3	D7 D8 D8	D7 D8 4
	10^{-7}	0 0 0	3 3 3	3 1 0	D10 D10 3	3 2 2
	10^{-8}		2 1 0	1 0 0	D8 D10 2	D4* 1 1
	10^{-9}		1 0 0	0 0 0	2 2 0	0 0 0
Complement Fixation	Undil.	0	0	++++	++++	++++
	½	0	0	+++	++++	++++
	¼	0	0	0	++++	++++
	⅛	0	0	0	+++	+++
	1/16	0	0	0	0	0
Flocculation	Undil.	0	0	±	+	++

*Eaten, not autopsied.

TABLE II.

Test	Dilution of fluid	WS-43 (extra-embryonic fluids) inoculated in dilution of: Undiluted	10⁻²	10⁻³	10⁻⁴	10⁻⁵	Normal extra-embryonic fluid
Time of harvest		24 hr	24 hr	36 hr	36 hr	44 hr	40 hr
Mouse Titration	10^{-7}	2 2 2	D6 D7 0	D6 D6 D9	D6 D7 D7	D6 D8 D9	
	10^{-8}	1 1 0	D8 D10 3	D8 D9 0	D7 D10 D11	D9 D10 D11	
	10^{-9}	0 0 0	D10 D11 3	D8 D9 3	D8 D8 0	D7 D8 D8	
	10^{-10}	0 0 0	4 3 3	D9 D11 3	D8 0 0	3 2 2	
Complement Fixation	½	++++	++++	++++	++++	++++	0
	¼	+++	++++	++++	++++	++++	
	⅛	0	0	++++	++++	++++	
	1/16	0	0	++++	++++	+	
	1/32	0	0	+++	++++	0	
	0	0	0	0	0	0	
Flocculation	Undil.	±	±	++	++	+	0

were collected correspondingly later. In all these fluids the infectivity for mice was practically identical, regardless of the concentration of the inoculum, excepting the preparation derived from eggs inoculated with the undiluted virus suspension. The low infectivity of this fluid, confirmed by similar experiments with the F-12 strain, has not yet been explained. The complement fixation antigen was present in high titer only in fluids collected from eggs injected with the 10^{-3} or 10^{-4} dilution which were harvested after 36 hours. Inoculation of the eggs with fluid in a dilution of 10^{-5} led to approximately the same virus titer, but less complement fixation antigen was found in the harvested fluid. The flocculation test corresponded closely to the complement fixation reaction.

It was found that under the conditions outlined above the allantoic fluid regularly contained complement fixation and flocculation antigen while the amniotic fluid frequently was negative and if positive, showed always a weaker reaction than the allantoic fluid. This fluid, on the other hand, in the presence of convalescent serum, frequently gave a complete fixation of complement in a dilution of 1:32. It can be heated to 56°C for 20 minutes, or dried from the frozen state and redissolved in distilled water, without serious loss of activity.

The pooled extra-embryonic fluids were used as antigen for the study of human sera obtained during a recent epidemic of influenza A in a closed institution. Some of the results are recorded in Table III, which shows a comparison between the flocculation, complement fixing and neutralizing antibodies of the sera of several patients taken (a) during the acute stage, and (b) 3 weeks after the

TABLE III.

Patient	Serum	Flocculation with extra-embryonic fluid: Influenzal	Flocculation with extra-embryonic fluid: Normal	Titer of compl. fixing antibodies	Titer of neutralizing antibodies	Virus isolated
B.S.	Acute	0	0	0	1:4	+
	Conval.	+	0	1:32	1:64	
F.B.	Acute	0	0	0	1:2	
	Conval.	+	0	1:32	1:128	n.t.
T.M.	Acute	0	0	1:2	n.t.	n.t.
	Conval.	+	0	1:16		
N.N.	Acute	0	0	0	n.t.	n.t.
	Conval.	+	0	1:16		
R.O.	Acute	0	0	0	n.t.	0
	Conval.	+	0	1:8		
K.T.	Acute	0	0	1:2	n.t.	0
	Conval.	±	0	1:8		
M.P.	Acute	0	0	1:2	1:16	0
	Conval.	±	0	1:4	1:64	
F.G.	Acute	0	0	1:2	1:4	n.t.
	Conval.	0	0	1:16	1:64	
M.M.	Acute	0	0	1:4	1:16	0
	Conval.	0	0	1:4	1:8	

n.t.—not tested.

onset of the disease. It is obvious from the table, that in all cases of influenza A showing a distinct rise in complement fixing and neutralizing antibodies, or from which the virus had been isolated (altogether 18 cases were observed) the flocculation was positive with one exception (F.G.). The flocculation seems to be correlated with the amount of complement fixing antibodies in that only sera with a titer of more than 1:8 showed a distinct flocculation while below this titer doubtful reactions, or none at all, were observed. One patient (M.M.) apparently did not suffer from influenza A since no rise in antibodies occurred, and correspondigly no flocculation was noticed.

Summary. The pooled extra-embryonic fluids of the chick infected with influenza A are very rich in virus. They can be used as an antigen for complement fixation when harvested at least 36 hours after inoculation of influenza A virus, the time for optimal yield depending on the dose of virus used as an inoculum. Furthermore, they show a flocculation phenomenon when mixed with convalescent or suitable immune sera. The allantoic fluid is a better antigen for the serological reactions than is the amniotic fluid.