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The Serological Differentiation of Mumps Complement-Fixation Antigens.*

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The recent adaptation of mumps virus to the chick embryo by Habel[1] and others[2,3] has greatly facilitated the serological diagnosis of the disease in its various manifestations and has placed such tests within the reach of the routine laboratory. The occurrence of a hemagglutination phenomenon similar to that observed with the influenza viruses permits the determination of antibodies by the inhibition of this reaction, as shown by Levens and Enders.[2] In addition, various materials derived from the infected chick embryo have been shown to contain complement-fixation antigen. Following the inoculation of the virus by different routes antigen was found in suspensions of the allantoic,[2] amniotic[2] and yolk sacs,[1-3] and in the allantoic[1] and amniotic fluids.[1,2] Studies in this laboratory, aside from confirming the earlier observations, were concerned with the demonstration of differences in the mumps-specific antigenic properties of suspensions of infected allantoic and amniotic sacs on the one hand, and of allantoic and amniotic fluids on the other.

Methods and Materials. A strain of mumps virus adapted to the amniotic sac of the chick embryo was kindly supplied by Dr. Enders. It has been propagated in this laboratory both in the amniotic and allantoic sacs of 8-day-old chick embryos for 8 to 14 consecutive passages, using for the transfers 10- to 10,000-fold diluted amniotic or allantoic fluids, respectively. For both routes a hole with a diameter of about 1 cm was cut with scissors into the shell over the air sac after the shell had been sterilized with 70% alcohol. In the case of the allantoic inoculation the needle was slipped just under the membrane to assure injection of the seed into the allantoic sac which is still very small on the 8th day of incubation of the embryo. For the amniotic inoculation a small part of the exposed shell membrane was removed over the approximate location of the embryo and the inoculum was deposited into the amnion under full visibility of this structure. The hole in the shell was sealed with scotch tape. After further incubation of the eggs at 35 to 37°C for 5 to 7 days, the virus usually attained maximal concentration in the respective embryonic fluids as measured by infectivity titrations in the chick embryo, hemagglutination and complement-fixation tests. At this time the allantoic and amniotic fluids and the corresponding membranes were harvested separately. The membranes were emulsified by means of a Waring blendor in saline solution to form a 10 or 20% suspension.

Infectivity titrations were performed in 8-day-old chick embryos by the injection of the virus preparation diluted in steps of 10 in broth by the allantoic (0.5 ml inoculum) or the amniotic routes (0.2 ml inoculum) depending on the passage series of virus used.

For the hemagglutination test 0.4 ml of virus suspension was mixed with 0.2 ml of a 1% suspension of washed fresh chicken red cells and the mixtures were incubated at 4°C until the erythrocytes had settled. The degree of agglutination was determined by the pattern formed by the cells at the bottom of the test tubes.

The complement-fixation technic was the same as employed for the studies on the antigenic differentiation of influenza antigens.[4] Dilutions of antigen, serum and

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1 Habel, K., *Pub. Health Rep.*, 1945, **60**, 201.

2 Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

3 Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 15.

4 Wiener, M., Henle, W., and Henle, G., *J. Exp. Med.*, 1946, **83**, 259.

guinea pig complement were mixed using 0.1 ml amounts of each. The mixtures were incubated at 37°C for one hour before the addition of 0.2 ml of sensitized sheep red cells (2.5% suspension). After further incubation for 20 minutes at 37°C and overnight at 4°C, the tests were read by estimating the degree of hemolysis obtained. Two units of amboceptor and 1.3 units of "Lyovac" complement† were employed. The highest initial dilutions of antigen or serum giving complete fixation of complement (no hemolysis) were recorded as titers. Most tests were performed according to optimal titration technic since zone phenomena were observed frequently in agreement with earlier observations by Enders and co-workers.[5] Human convalescent sera were used in these studies and the results were compared whenever possible with those obtained with sera from the same patients taken during the first days of the disease. Control antigens were prepared from suspensions of allantoic and amniotic membranes derived from normal embryos or from eggs infected with influenza B virus, in a manner corresponding to that used for the mumps preparations.

Experimental. Upon allantoic inoculation of mumps virus of the allantoic-passage series, complement-fixation antigen was found in the allantoic fluids and membranes and in the amniotic sacs, but none or little in the amniotic fluids. The allantoic tissue usually contained higher titers of antigen than the amniotic sac. Conversely, following amniotic injection of virus of the amniotic-passage series, higher titers of antigen were noted in the amniotic sac than in the allantoic tissue and, in early passages, the allantoic fluid gave negative or weak reactions in contrast to the strongly positive results with the amniotic fluids. Normal allantoic and amniotic fluids did not react with any of the sera used, whereas positive reactions with the suspensions of normal allantoic and amniotic membranes were obtained with some of the sera in low dilution. The antigen titers in the infected fluids, varying between 1:2 and 1:8, were lower than those observed in the corresponding membranes which were found to lie between 1:16 and 1:128. Hemagglutination tests, on the other hand, showed high titers in the fluids (1:256 to 1:1024 for allantoic, and 1:1024 to 1:8192 for amniotic fluids) but were low or negative with the suspensions of membrane. Although interpretation of the results of hemagglutination tests with the tissue suspensions has to take into consideration the inhibitory effect of some component in the emulsion, the data are comparable to those of infectivity titrations. In these the fluids revealed titers 100 to 1000 times higher than the suspensions of the membranes. The number of 50%-infectivity doses (ID_{50}) in the fluids varied from $10^{7.3}$ to $10^{8.8}$ per ml in the reported experiments, whereas the suspensions of membranes gave values between $10^{4.5}$ and $10^{5.8}$. These observations failed to show a correlation between the infectivity and hemagglutinin titers on the one hand, and the concentration of complement-fixation antigen on the other. This discrepancy has already been noted by Levens and Enders.[2]

These data suggested further comparisons among the various materials in regard to the nature of the antigens they contained. By the use of high speed centrifugation it could be shown that the mumps antigens of allantoic and amniotic fluids were sedimented together with the virus particle at 20,000 r.p.m. for 20 minutes. The infective, hemagglutinating and complement-fixing properties were found in the sediment whereas the supernatant fluid gave negative complement-fixation tests and only low titers of virus and hemagglutinin. This is in agreement with the findings of Beveridge and Lind.[6] From the tissue suspensions, on the other hand, only part of the complement-fixation antigen was removed by this procedure although the infectivity of the supernatant fluid showed a decrease in titer comparable to that observed with infected allantoic and amniotic fluids. The hemagglutination re-

† Generously supplied by Sharp and Dohme, Inc.

[5] Enders, J. F., Cohen, S., and Kane, W. W., *J. Exp. Med.*, 1945, **81**, 119.

[6] Beveridge, W. I. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 127.

TABLE 1.
Differential Centrifugation of Allantoic Fluid and Membrane Suspension Infected with Mumps Virus.

Preparation	Allantoic fluid				Allantoic membrane			
	Infectivity (allantoic inoculation), ID_{50}/ml	Hemagglutination titer	Complement-fixation maximal titer		Infectivity (allantoic inoculation), ID_{50}/ml	Hemagglutination titer	Complement-fixation maximal titer	
			Antigen	Serum			Antigen	Serum
Original	107.5	1:1536*	1:8†	1:32†	105.5	0	1:64	1:32
20,000 r.p.m. supernate	105.3	1:8	0		102.6	0	1:32	1:32
20,000 r.p.m. sediment 2 × conc.	108.3	1:4096	1:16	1:64	105.3	1:32	1:16	1:64
30,000 r.p.m. supernate	103.1	0	0		102.8	0	1:4	1:32
30,000 r.p.m. sediment 2 × conc.	108.3	0	0		101.8	0	1:16	1:32

* Initial dilution of the preparation giving last definite agglutination of red cells.
† Initial dilution of serum or antigen giving last complete fixation of complement.
All serum and antigen controls, as well as controls with acute serum and with normal membrane preparation gave negative results.

action, which usually was negative in the original suspensions, became distinctly positive in the resuspended sediments. This finding may be explained by the assumption that the hemagglutinins have been separated by the centrifugation procedure from an inhibitor present in the original suspension.‡ Recentrifugation of the 20,000 r.p.m.-supernate at 30,000 r.p.m. for one hour removed a large part of the "soluble" antigen, but some of it still remained in suspension. An experiment of this type, employing infected allantoic fluids and the corresponding membranes as the source of antigen, is shown in Table I. As pointed out, control antigens prepared from normal allantoic and amniotic sacs gave positive reactions with a few of the sera in low dilution. The material showing this reactivity was in part sedimented at 20,000 r.p.m., the supernatant being usually inert. It is obvious then that tissue antigens for diagnostic tests should be subjected to high speed centrifugation in order to avoid some of the nonspecific reactions.

The centrifugation experiments showed that physical differences existed between the antigen present in the embryonic fluids and at least part of the antigen present in suspensions of the membranes. In further experiments comparisons were made between preparations of infected allantoic fluid on the one hand, and the supernatant fluid after centrifugation at 20,000 r.p.m. of suspended allantoic membrane on the other; the former representing mostly large antigen particles which are sedimentable with the virus activity, the latter containing a smaller antigen not directly linked with the infective property. These comparisons showed on occasion differences in the optimal titration patterns of the 2 antigens but not as frequently and as markedly as was noted in the case of the corresponding influenza antigens.[4,7] More convincing evidence of serological differentiation was obtained by absorption of convalescent sera with the 2 types of

‡ Similar results were obtained upon dialysis of suspensions of infected membranes.

7 Henle, W., and Wiener, M., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 176.

TABLE II.
Results of Serum Absorption Tests.

Serum absorption				Optimal complement-fixation reaction Antigen							
				Allantoic fluid		Allantoic membrane					
							20,000 R.P.M.		30,000 R.P.M.		
Material	ID_{50}/ml serum	Units of antigen/ml serum	Dilution of serum	Orig. und.	20,000 R.P.M. sediment und.	Orig. 1:16	Supernate 1:16	Sediment 1:2	Supernate 1:4	Sediment 1:8	Saline
—	—	—	1:8	0	0	0	0	0	0	0	c
			1:16	0	0	0	0	0	0	0	c
			1:32	0	0	0	0	0	0	0	c
			1:64	tr	tr	wk	wk	0	st	tr	c
Allantoic fluid	$10^{9.4}$	320	1:8	st	st	0	0	0	0	0	c
20,000 R.P.M. sediment			1:16	c	c	0	0	0	wk	0	c
			1:32	c	c	ac	ac	ac	c	ac	c
			1:64	c	c	c	c	c	c	c	c
Allantoic membrane	$10^{5.9}$	1280	1:8	0	0	c	c	c	c	c	c
20,000 R.P.M. sediment			1:16	0	0	c	c	c	c	c	c
			1:32	ac	tr	c	c	c	c	c	c
			1:64	c	c	c	c	c	c	c	c
Allantoic membrane	$10^{2.2}$	320	1:8	0	0	c	c	st	c	c	c
30,000 R.P.M. sediment			1:16	0	0	c	c	c	c	c	c
			1:32	0	0	c	c	c	c	c	c
			1:64	ac	tr	c	c	c	c	c	c

0—no hemolysis; tr—trace; wk—weak; st—strong; ac—almost complete; c—complete hemolysis.

TABLE III.
Antibody Content of Various Selected Human Sera.

Case No.	Age (years)	History	Time after onset of disease	Serum antibody titer against: Virus-bound antigen (allantoic fluid)	Soluble antigen (Supernate 20,000 R.P.M. of allantoic membrane)	Normal allantoic membrane
1	4½	mumps	3 days	1:4	1:16	1:2
			15 "	1:64	1:64	1:2
			29 "	1:128	1:128	1:2
			46 "	1:128	1:64	1:2
2	5½	"	7 "	1:16	1:16	0
3	4	"	7 "	1:16	1:32	0
			17 "	1:64	1:64	0
4	6½	"	4 "	1:16	1:8	0
			14 "	1:64	1:32	0
6	4	"	4 "	1:4	1:8	<1:4*
			16 "	1:32	1:64	<1:4
36	9	"	2 months	1:64	1:8	0
35	7	"	3 "	1:32	1:8	0
31	8	"	2 years	1:8	0	0
28	2	"	2 "	1:8	0	0
11	4	"	2 "	1:4	0	0
25	10	"	2 "	1:8	0	0
13	11	"	2 "	1:4	0	0
44	9	unknown	—	1:16	0	0
43	6	"	—	1:8	0	0
42	9	"	—	1:8	0	0
19	8	inapparent mumps	16 days†	1:128	1:32	0
16	6	" "	39 " †	1:16	1:4	0

* Partial fixation of complement in serum dilution 1:4.
† Negative serum obtained 16 and 39 days, respectively, prior to the recorded specimen.

antigen. Human convalescent serum was absorbed with the sediments obtained by centrifugation at 20,000 r.p.m. from infected allantoic fluid or suspension of allantoic membrane, or with the material sedimented at 30,000 r.p.m. from the suspension of membrane after removal of the larger particles at 20,000 r.p.m. The ID_{50} for chick embryos as well as the units of antigen used for the absorption per 1 ml of serum were determined and are recorded in Table II. When mumps convalescent serum was absorbed with the antigen sedimented at 20,000 r.p.m. from allantoic fluid (virus particles) it no longer reacted with this antigen nor with the original allantoic fluid. It gave, however, distinctly positive reactions with suspensions of infected membrane and the fractions derived therefrom, although a decrease in serum titer was noted. On the other hand, absorption of the serum with the smaller antigen sedimented at 30,000 r.p.m. from suspensions of allantoic membrane previously cleared by centrifugation at 20,000 r.p.m. removed the reactivity with the "soluble antigen" but left antibodies to the allantoic fluid antigen and the sediment derived therefrom. Absorption of the serum with the particles sedimented from the membrane suspension at 20,000 r.p.m. gave results corresponding to the effect of absorption with the soluble antigen rather than with the virus particles obtained from allantoic fluid. The reasons for this latter finding are not clear as yet. The infectivity titrations of the suspensions of membrane used for the absorption showed only 0.1% of the activity of the allantoic fluid material. Consequently, there might not have been a sufficient concentration of virus to remove the antibodies to the virus-bound antigen. On the other hand, antibodies against the soluble antigen were completely removed by the particles sedimented from the membranes at 20,000 r.p.m. It is possible then that the "soluble antigen" occurs in different physical states, *i.e.*, in different particle sizes either *per se*, or as a result of adsorption onto par-

ticulate components of the host cell. One also might consider the possibility that the virus may occur in a different phase in the tissues, *i.e.*, covered with the soluble antigen, as compared to the "naked" virus in the allantoic fluid. This problem requires further investigation. Control absorptions of the convalescent serum with fractions sedimentable at 20,000 and at 30,000 r.p.m. from normal allantoic membranes or from tissues infected with the Lee strain of influenza B virus did not affect the reactivity of the serum with either the mumps virus as contained in the allantoic fluid or the mumps soluble antigen present in the suspension of membrane.

Further evidence of the serological differentiation between the 2 antigens was obtained in the study of the reactivities of various human sera with these preparations. Marked titers in antibodies against both antigens were found in sera of children convalescent from mumps and those having undergone a recent inapparent infection. Some sera from individuals with a history of long past infection or from subjects with unknown exposure showed antibodies only against the virus-bound antigen but not against the soluble material. Some of these cases are listed in Table III. This finding emphasizes again the difference between the 2 antigens; the one dominantly present in the embryonic fluids, the other in the suspensions of infected tissues. These results suggest, in addition, that the antibodies against the virus-bound antigen persist for a longer period of time following the infection than antibodies against the soluble substance. This, however, can be verified only by following the concentration of the 2 antibodies in individual patients over a prolonged period of time. In a few cases it was noted, too, that the antibodies to the soluble material may become measurable and reach higher concentrations earlier than the antibodies to the virus-bound antigen.

The demonstration of 2 distinct complement-fixing antibodies, and particularly the fact that one of them, the antibody against the soluble substance, may be absent from certain sera tends to explain discrepancies in the correlation of the results of complement-fixation tests with the susceptibility of an individual. If only tissue extracts are employed as antigen which contain mainly the soluble material a negative test may be recorded in a subject with a history of mumps at some time in the past. The use of the virus-bound antigen may properly classify such individuals. This latter antigen will also demonstrate past contact with the virus even though the history may not reveal past apparent infection.

The relation of the antibodies against the virus-bound antigen to the neutralizing and hemagglutination-inhibiting antibodies is under investigation at present. A comparison of the results of complement-fixation tests with these 2 antigens and skin tests is indicated.

Summary. Study of the complement-fixation antigens obtained from chick embryos infected with mumps virus revealed 2 antigenically distinct components separable by high speed centrifugation. One antigen is closely linked with the virus and found predominantly in the allantoic and amniotic fluids; the other component is smaller and present mainly in the allantoic and amniotic membranes. The 2 antigens can be differentiated by serum absorption technic. Some human sera contain antibodies to the 2 antigens (early convalescent sera) whereas others may show antibodies only to the virus-bound antigen but not to the soluble material (long past infection).