STUDIES OF THE HEMOLYSIS OF RED BLOOD CELLS BY MUMPS VIRUS*

I. The Development of Mumps Virus Hemolysin and Its Inactivation by Certain Physical and Chemical Agents

By LIANG-WEI CHU, M.D., and HERBERT R. MORGAN, M.D.

(From the Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor)

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Certain properties of a hemolysin associated with mumps virus have been described in a previous report (1). The labile nature of this hemolytic principle was indicated by its inactivation by heat and by its deterioration during storage under a variety of conditons. In order to facilitate further investigation of this viral hemolysin, it was necessary to determine the conditions for growth of mumps virus in embryonated eggs which would provide infected, extra-embryonic fluids with maximal hemolytic activity and, to ascertain the stability of the hemolysin under a variety of conditions. In this connection, the effects of certain physical and chemical agents on the hemolytic, hemagglutinative, and infective capacities of the virus were studied. These experiments would appear not only to indicate the stability of these three properties under a variety of conditions, but also by establishing the relative sensitivity of each to a given agent, may indicate certain relationships between them.

As used here, the term "hemolysin" indicates a virus particle containing material which produces hemolysis, without any implication that the active factor is a separable product rather than a constituent of the virus particle itself.

Materials and Methods

Virus.—The egg-adapted strain of mumps virus employed in a previous study (1) was used in the inoculation of fertile White Leghorn eggs which had been incubated for from 6 to 8 days at 39°C. Amniotic or allantoic fluid infected with mumps virus was diluted with beef heart infusion broth containing 10 per cent normal horse serum prior to inoculation of the eggs. For amniotic or allantoic inoculation, 0.1 ml. and for yolk sac inoculation 0.5 ml. of the inoculum was used. After incubating at 35°C. for 5 days, the amniotic and allantoic fluids were harvested separately. Aliquots were placed in glass ampoules and sealed, and stored at −70°C.

Virus Hemagglutination Titrations.—A pattern test using 0.25 per cent chicken red cells (2) was used with 0.85 per cent NaCl solution buffered at pH 7.2 (0.025 M phosphate) as the diluent.

Adsorption and Elution Technique.—One volume of a dilution of virus-infected fluid was mixed with 2 volumes of 10 per cent chicken erythrocytes. After adsorption at 4°C. for 1

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hour, the cells were sedimented by centrifugation and the supernatant fluid tested for hemagglutinins. The cells were resuspended in phosphate-buffered saline and incubated at 37°C. for 3 hours. The virus hemagglutinin content of the eluate was then determined.

Virus Hemolysin Titrations.—A method previously described (1) was used with some modifications. One ml. of serial twofold dilutions of the infected egg fluids in phosphate-buffered saline was mixed with 1 ml. of a 2 per cent suspension of chicken erythrocytes. The tubes were placed immediately in a water bath at 37°C. for 3 hours, at the end of which time 3 ml. of cold buffered saline was added. After inverting the tubes to assure thorough mixing, the cells were sedimented by centrifugation. The supernatant fluid was then transferred to a colorimeter tube and the hemoglobin content determined. The per cent of hemolysis was calculated with reference to a standard prepared by hemolyzing 1 ml. of the red cell suspension by the addition of 4 ml. of distilled water and a drop of dilute ammonium hydroxide. A tube containing 1 ml. of the red cell suspension and 4 ml. of buffered saline was included as a control of the stability of the red cells. The supernatant fluid from this tube was used for setting the zero point on the colorimeter. All determinations were made using a Klett-Summerson photoelectric colorimeter with a green filter Number 540.

Virus Infectivity Titrations.—Serial tenfold dilutions of the infected egg fluids were made in the 10 per cent serum broth and inoculated into the allantoic sac of 8 nine-day-old chick embryos. After 6 days at 35°C., allantoic fluid was withdrawn, diluted with an equal volume of buffered saline, and tested for its capacity to agglutinate chicken erythrocytes. Fifty per cent end points for virus infectivity (E.I. 50) were calculated according to the method of Reed and Muench (3).

EXPERIMENTAL

Factors in the Production of the Hemolysin

Route of Inoculation.—Three routes, namely, amniotic, allantoic, and yolk sac, were tested to determine their influence on the appearance of hemolysin. In embryos infected through the amniotic route virus was usually present in both amniotic and allantoic fluids. Hemolytic activity could be demonstrated in both fluids, though the amniotic fluid was usually much the more active (Table I). In embryos infected by allantoic inoculation, virus was present in the allantoic but usually absent from the amniotic fluid. Allantoic fluid from these embryos usually had low hemolytic activity. The results obtained in hemolysin tests with allantoic and amniotic fluids harvested after yolk sac inoculation from individual eggs showed great variation in potency of the fluids. In a few experiments, virus had not appeared in the amniotic or allantoic fluids of some embryos 5 or 6 days after yolk sac inoculation.

There seemed to be no relationship between the hemolytic titer of the seed virus used for inoculation and the hemolytic potency of the fluids subsequently harvested.

Age of Embryo and Time of Incubation.—Six to 8 day embryos were used for amniotic passage since with older embryos, the yield of amniotic fluid was much reduced at the time of harvest. Eggs usually began to die of virus infection on the 5th day after inoculation. The fluids from dead embryos generally had less hemolytic activity than those from surviving embryos. Hence

it was desirable to harvest the fluids not later than the 5th day at which time most of the embryos were still alive. Amniotic fluids from embryos inoculated on the 6th or 8th day of incubation showed good hemolytic activity but the large numbers of early deaths from trauma among the 6-day-old embryos made them unsuitable for use.

Virus Preparation and Hemolytic Activity.—Irrespective of the route of inoculation, the hemolytic activity of the infected allantoic fluid was generally poor. Even in eggs inoculated by the amniotic route, the hemagglutination titer of the allantoic fluid was, however, usually as high as, or only slightly lower than, that of the amniotic fluid (Table I). This suggested either that the virus present in the allantoic fluid had less hemolytic activity or that this fluid contained

TABLE I

Titration of Hemolytic Activity of Amniotic and Allantoic Fluids Obtained from Embryos Infected through the Amniotic Route

Fluids infected with mumps virus	Hemagglutination titer*	Hemolysis						
		Dilutions of fluid						Buffer control
		8	16	32	64	128	256	
Pool A								
Amniotic fluid	2048	52‡	51	38	24	15	10	0
Allantoic fluid	2048	36	23	12	9	4	3	0
Pool B								
Amniotic fluid	1024	39	38	23	14	5	4	0
Allantoic fluid	1024	8	7	4	2	0	0	0

* Expressed as the reciprocal.

‡ Per cent hemolysis.

a potent inhibitor of this hemolytic property, perhaps an excess of virus which had lost its hemolytic activity and subsequently acted as an interfering agent in the hemolytic process (4).

Emulsions prepared by grinding up the infected amniotic membranes in buffered saline had high hemagglutination titers but no hemolytic activity, in confirmation of unpublished observations of Enders and his associates (5). This finding might be explained by a difference in the properties of the virus mechanically released by disrupting infected cells, or by the presence of an inhibiting substance in the macerated tissues.

Effect of Certain Physical and Chemical Agencies on the Hemolysin

Effect of Storage at Various Temperatures.—At any of the temperatures used for storage of the virus preparations, the hemolytic property deteriorated long

before a reduction in the hemagglutinating capacity was evident. Infected amniotic fluid kept in the dry-ice cabinet without the addition of serum retained its hemolytic activity undiminished for about a month. Gradual reduction of the property then occurred and at the end of 4 months, it was barely detectable. When a marked decrease of hemolytic activity had occurred, the infectivity for embryonated eggs was also appreciably reduced. In one lot of amniotic fluid, the hemolytic activity in 1:8 dilution was 39 per cent after 1 month and 4 per cent after $4\frac{1}{2}$ months of storage in the dry-ice cabinet. The infectivity titers (E.I. 50) determined after the same intervals were $10^{-5.8}$ and $10^{-4.5}$ respectively.

TABLE II

The Effect of Freezing and Thawing on the Hemolytic Activity of Freshly Harvested Amniotic Fluids Infected with Mumps Virus

Fluids infected with mumps virus	Hemagglutination titer*	Hemolysis						
		Dilutions of fluid						Buffer control
		8	16	32	64	128	256	
Amniotic fluid A								
(*a*) Before freezing	1024	23‡	22	15	10	7	5	0
(*b*) After freezing for 24 hrs.	1024	59	53	44	28	17	11	0
Amniotic fluid B								
(*a*) Before freezing	2048	18	19	15	8	5	2	0
(*b*) After freezing for 2 hrs.	2048	30	32	24	17	12	6	0

* Expressed as the reciprocal.

‡ Per cent hemolysis.

At 4°C., a definite decrease in the hemolytic titer had appeared in 10 days but some activity was still demonstrable at the end of 3 weeks. A similar though more rapid change took place at room temperature (23–27°C.). At 35°C. deterioration occurred still more rapidly; the hemolytic activity was markedly reduced in 2 days and had almost disappeared in 3 days, while the hemagglutination titer remained unchanged.

Effect of Freezing and Thawing.—The hemolytic property of the amniotic or allantoic fluid was often found to be definitely increased after freezing and thawing (Table II). In these instances, no increase in hemagglutination titer, as determined by the pattern test, was observed. However, this enhancing effect of freezing and thawing was sometimes slight. Furthermore, the effect could be obtained only with freshly harvested fluids, not with fluids the hemolytic activity of which had deteriorated after long standing at room temperature or exposure to heat (50°C.). Freezing and thawing the emulsion of infected amniotic membranes did not result in its becoming hemolytic.

Effect of Heat.—In the earlier report by Morgan, Enders, and Wagley (1), it was noted that exposure of the infected fluids to heat at 50°C. for a short time produced marked reduction in the hemolytic property, while the hemagglutinating property was unimpaired. Experiments were performed with their technique, and in addition the E.I. 50 infectivity titers of the test fluids were determined to see whether there was any correlation between the loss of hemolytic activity and decrease of the infective capacity of the virus. The results of one experiment are presented in Table III.

The hemolytic property, as well as the infectivity, was reduced by exposure to 50°C., while the hemagglutination titer remained unchanged.

Adsorption-elution experiments on mumps virus heated to 50°C. for 30 minutes showed that marked reduction in hemolytic activity of the virus was not accompanied by any demonstrable loss of the ability of the virus to elute after its adsorption on red cells. The hemagglutinating capacity of the virus was

TABLE III

Effect of Heat upon the Hemagglutinating, Hemolytic, and Infectious Properties of Mumps Virus

Heat treatment of amniotic fluid infected with mumps virus	HA	HL	E.I.50*
Unheated	1024	56	$10^{-7.2}$
15 min. at 50°C.	1024	13	$10^{-5.8}$
45 min. at 50°C.	1024	0	$10^{-4.7}$

HA, hemagglutination titer expressed as the reciprocal. HL, per cent hemolysis with 1:8 dilution of virus.

* 50 per cent infectivity titer for chick embryos.

completely destroyed at 56°C. Exposure to temperatures between 50° and 56° resulted in various degrees of reduction in hemagglutinating activity. This was accompanied by partial failure of elution.

Effect of Formaldehyde.—Formalin was diluted in phosphate buffer-saline to give concentrations of 0.1, 0.2, and 0.5 per cent. Equal volumes of each of these dilutions of formalin were added to a similar amount of amniotic fluid infected with mumps virus. The mixtures were kept at 9°C. At various intervals of time, aliquots were removed from each tube for hemolytic and hemagglutination tests (Table IV).

The concentrations of formalin of 0.2 per cent and 0.5 per cent used in this experiment are known to destroy the infectivity of the mumps virus within 24 hours (6). In the present work the hemolytic property was rapidly abolished at these concentrations, whereas the hemagglutination titer was unaffected. No impairment of the power of the virus to elute after adsorption on red cells was observed after exposure to 0.2 per cent formalin for 24 hours at 9°C.

In another experiment using 0.2 per cent formalin at 9°C., change in hemolytic activity was correlated with change in infectivity of the virus (Ta-

TABLE IV

Inactivation of the Hemolytic Property of Mumps Virus by Formaldehyde

Fluid infected with mumps virus	Time of exposure	Per cent formalin							
		0		0.1		0.2		0.5	
		HA	HL	HA	HL	HA	HL	HA	HL
Amniotic	10 min.	1024	44	1024	31	1024	17	1024	8
	6 hrs.	1024	47	1024	17	1024	6	1024	0
	20 hrs.	1024	49	1024	6	1024	0	1024	0

HA, hemagglutination titer expressed as the reciprocal. HL, per cent hemolysis in 1:16 dilution of virus.

TABLE V

Effect of 0.2 Per Cent Formalin on the Hemolytic and Infective Properties of Mumps Virus

Fluid infected with mumps virus	Time of exposure to 0.2 per cent formalin	HA	HL	E.I.50*
	hrs.			
Amniotic	0	1024	42	$10^{-6.8}$
	1	1024	19	$10^{-3.3}$
	24	1024	0	$<10^{-1}$

HA, hemagglutination titer expressed as the reciprocal. HL, per cent hemolysis in 1:16 dilution of virus.

* 50 per cent infectivity titer for chick embryos.

TABLE VI

Effect of Ultraviolet Irradiation on the Hemagglutinative, Hemolytic, and Infective Capacities of Mumps Virus

Fluid infected with mumps virus	Time of exposure to ultraviolet light	HA	HL	E.I.50*
	min.			
Amniotic	0	2048	66	$10^{-7.37}$
	2	2048	44	$<10^{-1}$
	5	2048	20	$<10^{-1}$
	10	1024	3	$<10^{-1}$
	15	1024	0	—

HA, hemagglutination titer expressed as the reciprocal. HL, per cent hemolysis in 1:8 dilution of virus.

* 50 per cent infectivity titer for chick embryos.

ble V). Both hemolytic activity and infectivity were completely gone after 24 hours.

Effect of Ultraviolet Irradiation.—A General Electric ultraviolet germicidal

lamp was used as the source of irradiation. Infected amniotic fluid was placed in small Petri dishes (4 cm. in diameter) in amounts just sufficient to make a thin layer over the surface (1.5 ml. per dish). The uncovered dishes were held at a distance of 14 cm. from the lamp for various periods of time, after which the hemagglutinin, hemolytic, and infective titers of the fluids were determined.

The results of a typical experiment are given in Table VI. The amount of irradiation sufficient to reduce the hemolytic activity below the level demonstrable by the test employed left the hemagglutinating property almost unaltered. However, the infectivity was much more readily affected than the hemolytic property. After exposure for 2 minutes, the infectivity was completely lost, while the fluid still possessed considerable hemolytic activity.

Effect of pH.—The effects of hydrogen ions on the hemolytic capacities of the virus were studied by mixing 1 volume of infected amniotic fluid with 7 volumes of phosphate or glycocoll buffers at various pH levels. The suspensions were neutralized before testing for hemagglutination and hemolysis. After exposure for 1 hour at 4°C., no unfavorable effect was observed between pH 4.5–9.0. Some impairment of both properties occurred beyond these limits, and below pH 3 and above pH 10.6 both properties were completely destroyed. However, their stability at various pH levels was distinctly different when tested after intervals of 1 to 14 days. The hemolytic property was most stable at pH levels near 7.0 but deteriorated more rapidly as the pH values were farther removed from neutrality. In contrast, the hemagglutinating capacity was quite stable for as long as 7 days at pH levels between 4.5 and 9.0.

The pH stability of the hemolysin corresponds quite closely with that recorded for the infectious capacity of the virus by Weil and his associates (7).

DISCUSSION

In the study of the hemolytic activity of extra-embryonic fluids of eggs infected with mumps virus, it has been shown that amniotic fluid harvested from eggs inoculated by the amniotic route possessed the greatest hemolytic activity. Allantoic fluids obtained from eggs inoculated by the allantoic or amniotic routes usually produced little hemolysis in spite of the fact that hemagglutination tests indicated that the virus content of these allantoic fluids was about the same as that demonstrable in amniotic fluids which had shown maximal hemolytic activity. Several factors may have a bearing in understanding this interesting difference:—

Normal allantoic fluids have been shown to exert a pronounced inhibitory. effect on the hemolysis and agglutination of erythrocytes by mumps virus (4). If the inhibitor was present in infected allantoic fluids, it might account, in part, for their low hemolytic activity. However, the continued presence of the inhibitor in the allantoic sac after growth of the virus appears unlikely because

infected allantoic fluids with little hemolytic activity had hemagglutination titers comparable to those found in infected amniotic fluids with high hemolytic capacities and, if the inhibitor had persisted, it should have decreased the hemagglutinating activity as well as the hemolytic capacity of the allantoic fluid.

The demonstration that heated mumps virus, which loses its hemolytic activity while retaining its capacity to agglutinate red cells, will interfere with hemolysis by fresh mumps virus (4), might provide an explanation. If allantoic fluid contains a larger proportion of partially denatured virus particles which have no hemolytic capacity, it would have a much lower hemolytic activity than infected amniotic fluid. Furthermore, the presence of inactive virus particles could explain the almost complete absence of hemolytic activity which is frequently observed with amniotic or allantoic fluids obtained from dead embryos after infection with mumps virus.

Additional experimental work will be required to establish the possible roles of these two factors in the understanding of the inconsequential hemolytic activity demonstrated by many allantoic fluids infected with mumps virus.[1]

The failure of emulsions of infected amniotic membranes, with relatively high hemagglutinin titers, to show any hemolytic activity may be due, in part at least, to the release of inhibitors of hemolysis from the tissues during grinding of the membranes.

These studies have shown that the hemolytic capacity of mumps virus can be decreased or destroyed by heat, ultraviolet irradiation, formaldehyde, and changes in hydrogen ion concentration under conditions which have little or no effect on the hemagglutinating capacity of the virus. The ability of the virus to elute from red cells was retained after heating or treatment with formaldehyde under conditions which markedly reduced or abolished the hemolytic action of the virus. It appears then that the hemolytic activity of the virus is a much more labile property than its ability to agglutinate or to elute from erythrocytes. Loss of hemolytic activity may be considered as an early phase of degradation of the virus, while failure to elute from erythrocytes and subsequent loss of ability to agglutinate red cells occur as further steps in the destruction of the virus by certain physical and chemical agencies.

In the study of the relationships of various properties of the virus, it was of interest to determine whether loss of hemolytic activity was relevant to the infectivity of the virus. Although treatment with heat or formaldehyde affected both properties, the inactivation differed in degree. Heating a virus-infected fluid at 50°C. for 45 minutes destroyed all detectable hemolytic activity while the infectious titer was reduced from $10^{-7.2}$ to $10^{-4.7}$. This may be

[1] Subsequent experiments have shown that allantoic fluids, after dialysis with phosphate-buffered saline in the cold, possess uniformly high hemolytic titers.

explained by a difference in the sensitivity of the tests for hemolytic and infectious capacities since perhaps relatively few virus particles may be required to establish infection whereas a considerable number may be necessary to produce visible hemolysis. It has been shown by other investigators (8) that freshly harvested egg fluids infected with mumps virus must possess a virus content, as measured by infectivity titrations, of the order of $10^{-4.3}$ or more E.I. 50 to produce visible hemagglutination, and, it is probable that an even greater virus content is necessary for hemolysis. On the other hand, formaldehyde appeared to have a more rapid effect on infectivity than on hemolytic activity. After the exposure of infected amniotic fluid to 0.2 per cent formalin for 1 hour, the infectious capacity of the virus was more markedly reduced than its hemolytic activity. The difference observed, however, might be due to an artefact since some of the formaldehyde was carried along with the material injected into the eggs for the infectivity titrations, where it could conceivably exert a continuing effect on inoculated virus thus further reducing its infectious capacity. It is of interest in the comparison of hemolytic and infective properties of mumps virus, that the range of stability of the hemolysin from pH 4.5 to 9.0 is similar to that reported for the infectious capacity of mumps virus by Weil and his associates (7).

Though the hemolytic capacity and infectivity of the virus appeared to be affected in a similar manner by heat, formaldehyde, and changes in hydrogen ion concentration, they differed sharply in their response to ultraviolet irradiation. This procedure destroyed the infectivity of the virus completely under conditions of exposure at which much of its hemolytic activity was retained.

The test for the presence of hemolysin appears to measure a much more labile property of the virus than do tests for hemagglutination or elution of virus from red cells, and it may therefore be more useful as an indicator of the early changes associated with degradation of the virus.

SUMMARY

The conditions for the production of extra-embryonic fluids with hemolytic activity from chick embryos infected with mumps virus have been investigated. Infected fluids with strong hemolytic activity can be obtained by harvesting the fluids of 6- to 8-day-old chick embryos inoculated by the amniotic route after 5 to 6 days' incubation at 35°C. Under such circumstances, the hemolytic capacity of amniotic fluids is usually much higher than that of the allantoic fluids.

The hemolytic activity and infectivity of the virus have been found to be reduced or destroyed by heat, formaldehyde, and ultraviolet irradiation under conditions which leave the hemagglutinating capacity practically unchanged. Ultraviolet irradiation appeared to have a greater deleterious effect on the infectivity of the virus than on its hemolytic capacity. The marked reduction or

destruction of hemolytic activity of the virus produced by certain treatments with these various agencies was not accompanied by loss of the ability of the virus to elute following its adsorption on red blood cells during the process of hemagglutination. This test for hemolytic activity, which measures a more labile property of the virus than do determinations of virus hemagglutination or virus elution, may be useful in detecting changes which occur early during degradation of the virus.

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