Inhibition of Mumps Virus Multiplication by a Synthetic Polypeptide.* (20514)

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Synthetic lysine polypeptides have been demonstrated to reduce tobacco mosaic virus infectivity(1), to inhibit the growth of influenza virus in the chick embryo(2), to inhibit the multiplication of a bacteriophage(3), and to protect embryonated eggs infected with several strains of infectious bronchitis virus and Newcastle disease virus(4). In the present communication it will be shown that synthetic lysine polypeptides markedly reduce the susceptibility of the chick embryo to infection with mumps virus. Inhibition of mumps virus multiplication in the allantoic sac was obtained when very small amounts of polypeptides were injected either in the allantoic sac or yolk sac. Striking reduction in virus proliferation was obtained when the polypeptide was administered either 1 hour before infection or as late as 36 hours after infection with mumps virus. Evidence is presented to show that the virus *per se* is not demonstrably inactivated by the lysine polypeptides.

Materials and methods. (a) *Virus.* The Enders strain of mumps virus† used in these studies has undergone an unknown number of passages in monkeys' parotid glands, and 12 amnion and 36 allantoic sac passages in embryonated eggs. The virus was cultivated in the allantoic sac of 7-day old embryos which were incubated at 37°C for 6 days after inoculation. The allantoic fluids were harvested after preliminary chilling at 4°C for several hours. Virus inoculum pools were prepared as a 10% solution of infected allantoic fluid in sterile horse serum (previously heated at 56°C for 30 minutes). The preparation was divided into several aliquots which were stored at −70°C. Individual aliquots were then used and discarded.

(b) *Polypeptides.* The lysine polypeptide hydrochlorides‡(5) were dissolved in physiological saline and these solutions adjusted to pH 6.3 with alkali. The glutamic acid polypeptide(6) was dissolved in an equivalent amount of alkali and the solution adjusted to pH 7.3. All solutions were passed through a Seitz filter and tested for bacterial sterility. The maximum non-lethal dose of the lysine polypeptides was about 1.0 mg per chick embryo and of the glutamic acid polypeptide greater than 20 mg per embryo.

(c) *Virus infectivity titrations.* Serial 10-fold dilutions of infected allantoic fluid were made in 0.85% saline and 0.1 cc amounts injected into groups of 4 eggs intra-allantoically. After 6 days incubation at 37°C, each allantoic fluid was removed after preliminary chilling and tested for its ability to agglutinate chicken erythrocytes. Virus infectivity titration end points (I.D. 50) were calculated by the 50% end point method of Reed and Muench(7).

(d) *Virus hemagglutination titrations.* Serial 2-fold dilutions of infected allantoic fluid were prepared in 0.85% saline, and to 0.5 cc of each dilution in 10 x 75 mm pyrex culture tubes was added 0.25 cc of a 1% suspension of washed chicken erythrocytes. The tubes were read after standing 1 hour at room temperature and the end point was taken as the highest dilution at which complete or partial agglutination of the red blood cells occurred.

(e) *Reproducibility of hemagglutination titration end points.* To evaluate accurately the significance of differences in hemagglutination titers between control and treated groups of embryos, it was essential to calculate the reproducibility of titers under uniform conditions. Therefore, the standard error of

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TABLE I. Reduction of Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryos Injected with Polylysine.

1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Hemagglutination titer* Individual allantoic fluids				Geometric mean	Log mean	Difference from control log
	E.I.D.†							
Saline	10	128	256	32	16	65	1.81	
.4 mg PL‡	"	0	0	0		0		1.81
2 mg PGA§	"	256	256	256	16	128	2.11	0
Saline	30	512	256	128	128	224	2.35	
.4 mg PL	"	4	0	0		2	.20	2.15
Saline	300	320	320	160	160	221	2.33	
.4 mg PL	"	0	32	32	2	7	.83	1.50
Saline	3000	128	128	64		103	2.01	
.4 mg PL	"	8	0	8		4	.60	1.41
Saline	30000	256	256	256	256	256	2.41	
.4 mg PL	"	8	16	8	0	6	.75	1.66
Saline	300000	64	256	256	256	170	2.23	
.2 mg PL	"	32	64	16	64	38	1.58	.65‖

* Expressed as the reciprocal.
† E.I.D. = embryo infectious doses.
‡ Polylysine of avg molecular wt 4900.
§ Polyglutamic acid of avg molecular wt 4800.
‖ Statistical analysis by t test shows that 0.65 log unit difference was highly significant and would occur by chance less than 1 out of 50 times.

the geometric means of the hemagglutination titers of 9 groups of 3 or 4 embryos infected with 30 infectious doses of mumps virus was determined. The standard deviation of the distribution of end points (geometric mean of each group of infected embryos) was 0.24 log unit.

Experimental. In all experiments, fertile White Leghorn eggs were employed. The concentration of virus in the allantoic fluids was measured by the hemagglutination technic 6 days after infection with the virus(7). Ginsberg, Goebel, and Horsfall(8) have shown that hemagglutination is caused by the mumps virus particle itself and that the hemagglutination titer is a direct measure of virus concentration.

(a) *Effect of polylysine on chick embryos infected with various levels of mumps virus.* The experiments presented in Table I were carried out to determine the extent to which the administration of polylysine would inhibit the multiplication of mumps virus in the chick embryo. Groups of 3 or 4 embryos were injected with 0.2 or 0.4 mg of polylysine intra-allantoically. Control embryos were injected with saline. Levels of mumps virus ranging from 10 to 300,000 infectious doses were then inoculated 1 hour later by the same route. After 6 days incubation at 37°C, the eggs were harvested and the hemagglutination titers of the individual allantoic fluids determined. It is evident from Table I that the mean hemagglutination titers of the allantoic fluids from each group of embryos which had received polylysine and had been subsequently infected with from 10 to 30,000 infectious doses of mumps were strikingly lower than the controls. At these levels of virus only 0.7-4% of the maximum concentration of virus was produced in the presence of polylysine as measured by hemagglutination. The multiplication of mumps virus was significantly reduced even when 300,000 infectious doses of virus were injected. Polyglutamic acid of the same molecular weight had no effect on mumps virus multiplication.

There was no demonstrable hemagglutination in many of the infected embryos treated with polylysine. It has been previously shown (8) that virus titers of the order of $10^{-4.80}$ or higher are necessary before hemagglutination is demonstrable with mumps virus. Thus the zero values for the hemagglutination titers of many of the polylysine treated embryos could represent virus titers values ranging from 0

TABLE II. Reduction of Both Hemagglutination and Infectivity Titers of Mumps Virus in Allantoic Fluid of Chick Embryos Injected with Polylysine.

1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Hemagglutination titer of allantoic fluid	Infectivity virus titration end point ID_{50}, log
.2 mg PL*	30 E.I.D.	0	−3.7
"	"	0	−2.7
"	"	0	−4.0
			−3.5 Avg
Saline	30 E.I.D.	1:256	−7.5
"	"	1:256	
"	"	1:128	
			−4.0 Difference

* Polylysine of avg molecular wt 4900.

TABLE III. Effect of Different Quantities of Polylysine on Hemagglutination Titer of Allantoic Fluids Obtained from Chick Embryos Inoculated with 30 E.I.D. of Mumps Virus.

1st inj., .1 cc intra-allantoic	2nd inj., .1 cc intra-allantoic (1 hr later)	Hemagglutination titer* of allantoic fluids	Difference from controls, log
Saline	30 E.I.D.	224	
.4 mg PL†	"	2	2.15
Saline	30 E.I.D.	130	
.04 mg PL†	"	0	2.11
Saline	30 E.I.D.	65	
.01 mg PL†	"	6	1.06
.001 mg PL†	"	100	0
Saline	30 E.I.D.	103	
.04 mg PL‡	"	0	2.01
.01 mg PL‡	"	2	1.73
.001 mg PL‡	"	19	.75

* Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation.
† Polylysine of avg molecular wt 4900.
‡ Polylysine of molecular avg wt 20000.

to $10^{-4.30}$. It is therefore likely that in many cases the values for the reduction in virus concentration produced by polylysine, as represented by differences between treated and control hemagglutination titers, are minimum values due to the averaging into the mean of the zero values on the hemagglutination titer scale. To elucidate this, a separate experiment was done in which both hemagglutination and infectivity titers were simultaneously determined. A difference in hemagglutination titers between treated and control embryos was log 2.17, representing a 150-fold decrease in virus concentration (Table II). However, the actual reduction in virus concentration produced by polylysine, as revealed by infectivity titrations, was log 4.0, or a 10,000-fold decrease in virus production. Therefore the inhibition of mumps virus multiplication is probably greater than here reported in many instances where zero hemagglutination titers were obtained. These results also clearly demonstrate that the reduction in the hemagglutination titers of polylysine treated embryos was not due to an inhibition of the hemagglutination reaction, but represents a true inhibition of virus multiplication.

(b) *Effect of different quantities of polylysine on mumps virus multiplication.* In Table III are summarized the results of experiments to determine the least amount of polylysine which would inhibit mumps virus multiplication. Levels of polylysine from 1 to 40 μg were administered to embryos subsequently treated with mumps virus. Polylysine of molecular weight 4900 was effective in inhibiting virus multiplication at the 10 μg level but not at the 1 μg level. Polylysine of molecular weight 20,000 was even more effective, inhibition of virus multiplication be-

TABLE IV. Effect of Time Interval between Injections of Mumps Virus and Polylysine.

1st inj., .1 cc intra-allantoic	Interval, hr	2nd inj., .1 cc intra-allantoic	Hemagglutination* titer of allantoic fluids Treated	Control	Difference from controls, log
.4 mg PL†	1	30 E.I.D.	2	224	2.15
30 E.I.D.	8	.4 mg PL	0	130	2.11
"	24	.2 mg PL	1	204	2.16
"	36	.2 mg PL	0	204	2.31
"	44	"	24	46	.35
"	52	"	5	46	.98
"	56	"	106	65	0
"	80	"	96	65	0

* Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation with virus.

† Polylysine of avg molecular wt 4900.

TABLE V. Effect of Injection of Polylysine into Yolk Sac upon Hemagglutination Titer of Mumps Virus in Allantoic Fluid of Chick Embryos.

1st inj., .1 cc	Time interval, hr	2nd inj., .1 cc	Hemagglutination titer* of allantoic fluids	Difference from controls, log
Saline (AS)†	1	30 E.I.D. (AS)	204	
.4 mg PL‖ (YS)‡	1	"	0	2.31
Saline (AS)	1	30 E.I.D. (AS)	100	
.2 mg PL§ (YS)	26	"	32	.45
.2 mg PL‖ (YS)	1	"	24	.62

* Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3 or 4 chick embryos obtained 6 days after inoculation.

† AS = Allantoic sac route.

‡ YS = Yolk sac route.

§ Polylysine of avg molecular wt 4900.

‖ " " " " " 20000.

ing obtained with only 1 μg of the polypeptide.

(c) *Effect of time interval between injection of mumps virus and polylysine.* Experiments were carried out to determine how long after inoculation of mumps virus polylysine was capable of causing inhibition of virus multiplication (Table IV). A single injection of 0.2 mg of polylysine, given 1 hour before or as late as 36 hours after inoculation of mumps virus, drastically inhibited viral multiplication. In all cases the differences between hemagglutination titers of allantoic fluids obtained from controls and those treated within 36 hours was over 2.0 log units. This reflects at least a 99.5% inhibition of viral multiplication. Inhibition by polylysine became less striking as the time interval was further increased, and no inhibition could be demonstrated when the polypeptide was inoculated 56 hours after infection with mumps.

(d) *Effect of injection of polylysine into yolk sac.* Experiments were carried out to determine whether injection of polylysine by a route other than the allantoic sac would also cause inhibition of mumps virus multiplication in the allantoic sac. The injection of 0.4 mg of polylysine, of molecular weight 4900, in the yolk sac 1 hour before inoculation of mumps virus in the allantoic sac completely inhibited hemagglutinin formation in the allantoic sac (Table V). However, polylysine of molecular weight 20,000 when injected in the yolk sac had much less inhibitory effect on the multiplication of mumps virus in the allantoic sac. It appears that the smaller polypeptide can more readily pass from the yolk sac to its site of action in the allantoic sac. When the smaller polypeptide was inoculated in the yolk sac 26 hours prior to injection of mumps virus, no significant inhibition of viral multiplication was obtained. Apparently, polylysine is metabolized, bound to protein, or removed in some manner from circulation within 26 hours after inoculation. This is supported by previous work which showed that polylysine cannot be demon-

TABLE VI. Effect of Polylysine on Mumps Virus *In Vitro*.

Mixture held at 3°C 30 min.		Constituents of inoculum			
Mumps virus dilution	Solvent	Mumps virus	PL. μg/embryo	Infectivity score	Virus titration end point ID_{50}, log
10^{-4}	Saline	10^{-6}	0	3/3	
"	"	10^{-7}	0	1/4	
"	"	10^{-8}	0	0/4	−6.75
10^{-4}	PL,* .5 mg/cc	10^{-6}	.5	3/3	
"	"	10^{-7}	.05	2/4	
"	"	10^{-8}	.005	0/4	−7.00

* Polylysine of avg molecular wt 4900.

strated in allantoic fluid by hemagglutination technic 24 hours after inoculation into the allantoic sac of chick embryos(9).

(e) *Effect of polylysine on mumps virus in vitro.* It was of interest to determine whether the very basic lysine polypeptide caused inactivation of the mumps virus particle. Hence, mumps virus was incubated with polylysine as indicated in Table VI, serial decimal dilutions of the mixture were then made with saline, and a virus infectivity titration carried out. As a control, the same procedure was repeated substituting saline for polylysine in the incubation mixture. Despite the presence of a high concentration of polylysine and a relatively low concentration of mumps virus in the incubation mixture, the infectivity titer of the virus after treatment with polylysine was not significantly different from the control, indicating that polylysine was not virucidal and that the effects observed *in vivo* cannot be readily explained by an inactivation of mumps virus by polylysine. However, these findings do not rule out the possibility of a reversible combination of polylysine with mumps virus in such manner as not appreciably to inactivate the virus.

Discussion. The evidence here presented demonstrates that polylysine inhibits multiplication of mumps virus in the allantoic sac of the chick embryo. These results are particularly striking since marked inhibition was obtained when large quantities of virus were given (*i.e.*, 300,000 infectious doses). Inhibition of viral growth was obtained with as little as 1 μg of polypeptide per embryo. It is important that the inoculation of polylysine as late as 36 hours after injection with mumps virus drastically inhibited viral multiplication, and that polylysine could be administered by a route other than that of virus inoculation; injection of a 4900 molecular lysine polypeptide in the yolk sac completely inhibited hemagglutinin formation in the allantoic sac.

Polylysine has been shown to be effective against a number of viruses in the chick embryo: influenza A, infectious bronchitis virus of chickens, Newcastle disease virus, mumps virus, and recently influenza B virus(10). The mechanism of action of polylysine in inhibiting viral multiplication deserves further consideration. The extreme reactivity of polylysine makes it difficult to discover precisely where in the chain of virus reproduction the polypeptide exerts its effect. It has been previously reported that polylysine will combine with red blood cells(9), tobacco mosaic virus(1), bacteria(11), nucleic acids(12) and many proteins(13). In view of this marked reactivity of the polypeptide, several possible modes of action of polylysine can be postulated: 1. The polypeptide might inactivate the virus particle *per se.* This is not supported by data here presented where it was shown that incubation of the virus with large quantities of polylysine did not demonstrably alter its infectivity. 2. Polylysine might combine with the virus particle extracellularly in a manner similar to neutralizing antibodies so that the virus cannot attach to the host cell. However, antibodies in general are completely ineffective in preventing viral proliferation once virus has penetrated the cell. In contrast, polylysine is effective when inoculated up to 36 hours after infection with mumps virus. Thus, polylysine inhibited viral multiplication when inoculated any time within the first cycle of multiplication and even into the second cycle of multiplication(14). In this respect, the action of polylysine in

inhibiting viral multiplication is different from that of antibodies. 3. Polylysine might combine with receptor cell sites and thus prevent virus-cell interaction. This is suggested by the fact that both virus and polylysine combine with and agglutinate red blood cells. However, there is no necessary correlation between the ability of polypeptides to inhibit viral proliferation and to combine with red blood cells, for polyglutamic acid, which also agglutinates erythrocytes(10), does not inhibit viral multiplication. It is important in this respect to point out that polylysine is effective as an inhibitor of mumps virus multiplication in the chick embryo when inoculated after virus adsorption is complete. There remains a possibility that virus-cell interaction might be prevented in the second cycle of multiplication of the virus. However, since polylysine is not effective when given 26 hours before virus it would seem that the polypeptide would not remain available in the embryo over the 32 hours required for a complete cycle of multiplication of mumps virus.

4. Polylysine might prevent release of new virus particles from infected cells. We have no experimental data bearing on this possibility. 5. Polylysine might act intracellularly by diffusing into the cell and combining with intact virus, virus fragments, or intermediates in virus formation within the cell. Combination with intermediates in virus synthesis would seem possible in view of the known reactivity of polylysine and the ease with which it combines with proteins, nucleic acid, and tobacco mosaic virus by formation of ionic bonds with acidic groups. Such a combination should obey the law of mass action and an equilibrium would be established between polylysine, virus-intermediate and the polylysine-virus-intermediate complex. If this were the case, increasing the amount of polylysine administered would reduce the concentration of free virus intermediate and virus multiplication would be correspondingly diminished. Likewise by increasing the virus inoculum or by delaying the injection of polylysine for sufficient time, the concentration of free virus intermediate would be increased and inhibition reduced. The results reported here are consistent with this explanation.

Virus inhibition by lysine polypeptides may involve more than a single mode of action. The biological effects of the polylysine are no doubt associated with its reactivity in combining with acidic groups. Whether these groups involved are on the surface of virus particles, virus intermediates, virus receptor sites or other critical substances necessary for virus formation cannot yet be decided. It is possible that such combinations may occur outside the cells, on the cell surface, or within the infected cell.

The antiviral activity of the lysine polypeptides is sufficiently high to warrant further investigation. These studies are being continued and the effects in experimental animals are now under study.

Summary. Lysine polypeptides cause marked inhibition of the multiplication of mumps virus in the allantoic sac of the chick embryo. Inhibition was obtained with as little as 1 μg of polypeptide. Polylysine was effective when injected as late as 36 hours after inoculation of virus. The polypeptide caused inhibition of viral multiplication in the allantoic sac when injected either in the allantoic sac or the yolk sac. Possible mechanisms of inhibition of viral multiplication are discussed.

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Thromboplastinase, a New Enzyme Which Destroys Thromboplastin. (20515)

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The nature of thromboplastin has long been a subject of investigation. This material is clearly described only in terms of its activity: the initiation of the blood coagulation process. The most active, best characterized thromboplastic preparation to date is the lipoprotein fraction obtained by differential centrifugation according to Chargaff(1). Whether thromboplastic activity is associated with a specific molecule, a type molecule, or a combination of factors awaits clarification. The nature of the active lipid moiety of thromboplastic preparations likewise remains obscure. The relationship between the active materials in thromboplastic suspensions prepared from different tissues, as well as that found in human blood is undetermined. The preparation, preservation, variation in activity and fundamental mode of action of this material consequently remain empirical and ill-understood.

This laboratory recently reported on the marked reduction of thromboplastic potency in tissue suspensions due to a bacterial contaminant(2). The organism was later found to accomplish this potency reduction by virtue of an adaptive enzyme which it elaborated. Bacteriological studies are presented elsewhere (3).

This communication will describe the preparation and properties of the new enzyme, tentatively called "thromboplastinase" which we believe will be a useful tool in the investigation of thromboplastin. Our studies have led to evidence for a structural moiety necessary for thromboplastic action which is common to thromboplastic suspensions of different origins.

Materials. Glassware. All glassware was scrupulously cleaned in detergent, dichromate cleaning solution and then thoroughly rinsed. *Plasma.* Normal human plasma was collected in 1/9 volume oxalate in glass test tubes, centrifuged, and the supernatant plasma removed. *Thromboplastin.* Rabbit brain(4), human placenta(5), or human brain(3) were used as sources of thromboplastin. *Thromboplastinase.* An adaptive enzyme elaborated by a *Bacillus cereus-B. megatherium* intermediate under certain conditions.†‡ This organism grew well in a simple medium of salts and sugar, without elaborating thromboplastinase. However, if the substrate was a biological source medium such as a thromboplastic tissue extract or Brain Heart Infusion, (Difco), the enzyme was formed. Growth and enzyme production proceeded as well when the dialysate of Brain Heart Infusion was used as the medium. The dialysate was therefore employed to avoid the extraneous protein of the Brain Heart Infusion. The following method was then used to obtain crude enzyme.

A Brain Heart Infusion dialysate was prepared by placing a dialysis bag containing 250 ml of 0.9% Brain Heart Infusion into 1 liter of distilled water in a 2 liter flask and autoclaving at 15 lb for 20 minutes. Upon cooling, the sterile dialysate was seeded with a 24-hour (37°C) culture from a Brain Heart Infusion-agar slant and allowed to incubate at 37°C for 24 hours. During the course of this incubation, a progressive increase in enzyme activity of the culture medium was

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† Previously mistakenly reported to be *B. subtilis*.

‡ Obtainable from ATCC as No. 10987 (NR Smith's strain 248).