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Reversible Inhibition of Poliovirus Ribonucleic Acid*

By

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Introduction

Since the extraction of infectious ribonucleic acid (RNA) from tobacco mosaic virus was achieved (1, 2) infectious RNA from a great many RNA-containing viruses has been obtained. The cell only needs to be infected with RNA from a virus to get the complete information for producing new virus particles. However, in most cases, the efficiency with which RNA is capable of entering the cell is much lower as compared with mature virus. Several procedures were developed to enhance the cell entering efficiency of infectious RNA, but it seems that one enhancing procedure applies only for a particular RNA cell system. In the case of poliovirus, there are two main enhancing or facilitating systems for RNA. One uses a hypertonic inoculum on hypertonically treated HeLa cells (3, 4). This procedure, however, does not enhance the infection of polio RNA in monkey kidney tissue culture (MKTC) cells, but raises the plating efficiency of mature poliovirus on MKTC up to two-fold (*Engler* and *Tolbert* unpublished data). The other procedure facilitates the infection of MKTC cells with polio RNA by adding any one of several insoluble substances to the inoculum, and by depleting the cell sheet of calcium prior to inoculation (5). The efficiency of infection obtained with RNA seems to depend largely on the physical and/or physiological environment in which the infection takes place. It also has been reported that there is an RNA inactivator at the surface of amnion cells which prevents the cells from being infected by RNA (6). For quantitative determination of infectious RNA, the necessity of obtaining more information on factors derived from the cells influencing the infectivity of polio RNA became apparent.

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Materials and Methods

Preparation of Infectious RNA.

7% Method: We used essentially the method described by *Klingler* et al. (7). 93 volumes of poliovirus suspension (Brunhilde, Type 1) plus 7 volumes of water saturated phenol were mixed vigorously for about five minutes at room temperature. The mixture was then extracted three to four times with ether to remove the phenol. The remaining ether was evaporated by bubbling air or nitrogen through the solution.

50% Method: The method of obtaining infectious RNA described by *Gierer* and *Schramm* (1) was used except that only one extraction with phenol was made. One volume of poliovirus solution and one volume of water saturated phenol were shaken for two minutes. The mixture was centrifuged five minutes at 1800 r. p. m. to separate the two phases. The water phase was collected and the remaining phenol was extracted as described above.

Infection of MKTC's with Infectious RNA and Development of Plaques.

MKTC monolayers, grown in 60 mm. petri dishes were infected with RNA as described by *Dubes* and *Klingler* (5). Briefly, the cells were washed four times with calcium free phosphate buffered saline (PBS) allowing them a depletion period of 30 minutes at 37° C between the second and third wash. As facilitator, kaolin (Fisher Scientific Co.) was used. A final concentration of 0.25% (w/v) of kaolin was present in every inoculum. To serial dilutions additional kaolin was added to keep its concentration at 0.25%. The RNA was allowed to adsorb onto the cell sheet for 30—50 minutes. The cells were then overlaid with high cystine altered Eagle's medium (hcaEM) (8) containing agar and maintained in a continuous CO_2 flow incubator at 36° C $\pm$1° C. After three days the plaques were made visible by staining the surviving cells with neutral red (9).

Results

Influence of MKTC Cell Homogenate on Infectious Polio RNA and Its Possible Ribonuclease (RNAase) Activity

Crude poliovirus preparations necessarily contain the debris of the cells on which the virus has been propagated. The method of preparing infectious RNA with 7 per cent phenol does not remove any of the cell components present in such crude harvests. To obtain information on whether or not cell homogenates affected infectious RNA, we tested their influence on polio RNA with the intent of determining possible RNAase activity. The cell homogenates were obtained by scraping off the cell sheets of uninfected petri dishes in 2.5 ml. of PBS. The harvests were homogenized for 2 minutes in a Potter teflon homogenizer (TRI-R. Co., Jamaica, N. Y.). From a stock of infectious RNA used for most of these experiments, 0.2 ml. was incubated for 30 minutes at 37° C with 1.6 ml. of uninfected cell homogenate and 0.2 ml. of 2.5% kaolin in PBS. For comparison the RNA was incubated at the same concentration under the same conditions with crystalline RNAase at 0, 0.1, 1, and 10 μg/ml. The incubated RNA solutions and ten and one hundred fold dilutions there from were plated. As mentioned in the "Materials and Methods" section, kaolin at a con-

centration of 0.25 per cent was used to facilitate the RNA; the kaolin concentration was kept constant in the several dilutions used. The results obtained are listed in Table 1. Despite some variation in plaque number at higher RNA concentrations which is observed frequently with RNA inocula, the results indicated clearly the diminishing effectiveness of RNAase with decreasing concentration. RNAase at a final concentration

Table 1. Inactivation of Infectious RNA by RNAase Compared with its Inhibition by Cell Harvest

Inoculum*	Arithmetic mean of the number of RNA plaques per petri at the concentration of:		
	1:1**	1:10	1:100
RNA without RNAase or cell homogenate	40.0	22	6
RNA incubated 30 minutes at 37° C without RNAase or cell homogenate	28.0	20	4
RNA incubated 30 minutes at 37° C with cell homogenate	1.5	10	n. d.***
RNA incubated 30 minutes at 37° C with 10μg RNAase/ml.	0	0	n. d.
Ditto but with 1 μg RNAase/ml.	20	11	0.6
Ditto but with 0.1 μg RNAase/ml.	40	11	6

* The RNA concentration was kept constant. In the absence of RNAase and/or cell homogenate, PBS was substituted.

** The ratio 1:1 means the starting inoculum concentration for all tests. In actuality this constitutes a ten-fold dilution of the RNA with facilitator, and inhibitor or PBS.

*** n. d. = not done.

of 10 μg/ml. destroyed the RNA completely, whereas at 0.1 μg/ml. we noted no decrease in plaque-forming capacity. The inactivation with 1 μg/ml. RNAase took an intermediate position, as noted particularly at the 1:100 dilution step.

The infectivity obtained from RNA incubated with cell homogenate took an unexpected pattern. The plaque number of the concentrated inoculum was lowered to an average of 1.5 plaques per petri; the cell homogenate had almost the same inactivation effect as 10 μg/ml. of RNAase. However diluting the concentrated inoculum ten-fold restored infectivity to that of the untreated RNA at the same concentration. The cell homogenate, therefore, did not inactivate the infectious RNA in the

same way as RNAase, but when present in sufficient quantity inhibited the RNA. The inhibitory effect was shown even more strikingly when an RNA solution inhibited by cell homogenate was diluted once in PBS and once in additional cell homogenate. The results of a typical experiment are listed in Table 2. The infectious RNA was again very strongly inhibited by the cell homogenate. If a cell homogenate suspension was used as diluent, the infectivity of the RNA dropped virtually to zero. However, diluted into PBS, RNA was restored to its original infectivity.

Inhibition of Infectious RNA by Denatured Cell Homogenate and Components

The cell homogenate was fractionated by centrifugation, to determine the location of the inhibitor. The following steps were used. Large cellular debris, and nuclei were removed from the suspension by ten minutes

Table 2. Effect of Diluting RNA Incubated with Cell Harvest into PBS or Additional Cell Suspension

Inoculum	Arithmetic mean of the number of RNA plaques per petri at the concentration of:		
	1:1	1:10	1:100
RNA control in PBS	51	15.0	3
RNA incubated with cell homogenate	2		
Ditto, diluted into additional cell homogenate		0.3	0
Ditto, diluted into PBS		12.0	3

centrifugation at 2500 r. p. m. (low speed sediment). From the supernatant medium size particles were removed by 29 minutes centrifugation at 30,000 r. p. m. in a No. 40.0 head of a Model L Spinco centrifuge (medium speed sediment). From the supernatant of the second centrifugation, smaller particles including much of the ribonucleoprotein (RNP) were obtained by centrifugation at 40,000 r. p. m. for 65 minutes with the same rotor in the same centrifuge (high speed sediment). All three sediments obtained were restored with PBS to the original volume of cell homogenate from which each was obtained. The resuspended sediments and the supernatant of the last centrifugation (high speed supernatant) were tested for their inhibitory effect. Nitrogen determinations of the four fractions were made by the Kjeldahl method to determine the nitrogen content of the different fractions. Table 3 shows the inhibitory effect of the four cell fractions as well as the amounts of nitrogen containing cell material accumulated in these fractions. Only the high speed supernatant and the low speed sediment, where about one-half and one-third respectively

of the nitrogen containing cell material was present, inhibited the RNA similarly to the complete cell harvest. The medium-speed sediment inhibited the RNA to a small degree, whereas the high-speed sediment had no inhibitory effect. Each of these two fractions contained less than 10 per cent of the nitrogen containing cellular material. Correlating the amount of material recovered in the fractions with their inhibitory effect, suggested

Table 3. Inhibition of Infectious RNA by Cell Components

Inhibitor	Treatment and diluents	Arithmetic mean of the number of RNA plaques per petri at the concentration of:		µg nitrogen per ml. of inhibitor solution
		1:1	1:10	
None	RNA control in PBS	24	13	—
Complete cell homogenate	RNA incubated with inhibitor further diluted into inhibitor further diluted into PBS	6	0.5 7	83.0 (100%)
Lowspeed sediment (cell debris and nuclei)	ditto	1	2 7	27.4 (33%)
Med. speed sediment (mitochondria)	ditto	13	2 9	7.7 (9.3%)
High speed sediment (RNP* particles)	ditto	40	13 10	6.8 (8.2%)
High speed supernatant (soluble cell components)	ditto	2	0.5 12	41.8 (50.4%)

* RNP = Ribonucleoprotein.

that differences in inhibition were caused by different concentrations of nitrogen-containing cell components and could not be attributed to a particular cell fraction.

When poliovirus RNA is prepared by the 7 per cent method, the cellular protein material remains in the RNA solution; however, one can assume that much of it is denatured by the phenol. We investigated, therefore whether after treatment with 7 per cent phenol, or after heating the cell harvest to 100° C for 15 minutes, the inhibitory capacity of the cell homogenate remained.

The results are listed in Table 4. It is obvious that neither of the two denaturing treatments changed the inhibitory effect of the cell homogenate. Therefore one can discard the possibility that the inhibition is based on a biological reaction between the cell homogenate and the RNA which could be stopped by denaturing.

Inhibitory Effect of Non-specific Proteins, Media, Commercial RNA, and DNA on Infectious Polio RNA

As indicated in experiments described above, the inhibition effect of cell homogenate on infectious RNA is a function of the concentration and cannot be related to any cellular components. A number of substances

Table 4. RNA Inhibition by Phenol and Heat Treated Cell Homogenate

Inhibitor	Treatment and diluents	Arithmetic mean of number of RNA plaques obtained per petri dish at the concentration of:		
		1:1	1:10	1:100
None	RNA control in PBS	51	15	3
Untreated cell homogenate	RNA incubated with inhibitor	2		
	further diluted into inhibitor		0.3	0
	further diluted into PBS		12	5
Phenol-treated cell homogenate	ditto	3	0.6	0
			21	5
Heat-treated cell homogenate	ditto	4	0.6	0
			16	2

were tested for their inhibitory effect on infectious RNA such as: yeastolate (Difco), eggwhite, casein hydrolysate, commercial RNA, and commercial desoxyribonucleic acid (DNA; Nutritional Biochemical Company). The two media used for growth of MKTC's and virus respectively: lactalbumin growth medium (LG) and hcaEM were also tested for inhibition of infectious RNA. The concentrations of the protein containing compounds, RNA, and DNA were chosen to be in the same magnitude as the concentration of cell homogenate previously tested. From nitrogen and dry weight determinations of the cell homogenate values were obtained for substitution of other substances in place of the homogenate. These substances were present at a concentration of 1.2 mg./ml. and the nucleic acids (NA's) at 0.3 mg./ml. In Tables 5 and 6 the inhibitory effects of the

Table 5. Inhibitory Effect on Infectious RNA of Some Protein Containing Compounds, hcaEM and LG

Inhibitor	Treatment and diluents	Arithmetic mean of the number of RNA plaques per petri at the concentration of:	
		1:1	1:10
None	RNA in PBS	28	15
Yeastolate	RNA incubated with inhibitor further diluted into inhibitor further diluted into PBS	9	 2 23
Egg white	ditto	5	0.6 10
Casein hydrolysate	ditto	6	0.6 17
hcaEM*	ditto	37	16 14
LG**	ditto	1	0 4

* High cystine altered Eagle's medium.
** Lactalbumin growth medium.

Table 6. Inhibitory Effect of RNA and DNA on Infectious RNA

Inhibitor	Treatment and diluents	Arithmetic mean of the number of RNA plaques per petri at the concentration of:	
		1:1	1:10
None	RNA in PBS	ca. 100	36
RNA	inf. RNA incubated with inhibitor further diluted into inhibitor further diluted into PBS	10	 2 37
DNA	ditto	13	5 22

protein containing compounds, media, and NA's are listed. All the substances and NA's tested had inhibitory effects similar to that of the cell homogenate. The inhibition was reversible when the inhibited RNA was diluted into PBS. The effect of the two media tested, however, was different. The hcaEM did not inhibit the infectious RNA, however, the LG inhibited and inactivated the infectious RNA. The inactivation of RNA

by LG is obvious if one compares the four plaques/petri obtained after diluting the treated RNA into PBS with the approximately 15 plaques/petri obtained in the control or PBS dilution of the inhibited RNA. The destruction of infective RNA by LG can probably be attributed to the presence of serum and perhaps RNAase in this medium.

The same substances and NA's used above to inhibit infectious RNA did not inhibit infection with mature virus when tested under the same conditions.

Table 7. Inhibitory Effect of Cell Harvests Previously Treated with Kaolin on Infectious RNA

Inhibitor	Treatment and diluents	Arithmetic mean of the number of RNA plaques per petri at the concentration of:	
		1:1	1:10
None (control 1)	RNA in PBS	31	14
Cell harvest (control 2)	RNA incubated with inhibitor	2	
	further diluted into inhibitor		0
	further diluted into PBS		12
Cell harvest treated once with 2.5% kaolin	ditto	15	6 17
Cell harvest treated twice with 2.5% kaolin	ditto	30	14 12

Mechanism of Inhibition

The infection of MKTC cells with RNA facilitated with kaolin (or any other facilitator) can be subdivided into two steps. The adsorption of the RNA to the facilitator takes place first, then the RNA-facilitator complex infects the MKTC cell rendered more susceptible by calcium depletion.

MKTC's were regularly depleted of calcium. Some of the MKTC's were maintained for an additional 15 minutes under 2 ml. of Ca^{++}-free PBS at 37° C. The other part was kept for the same time at the same temperature under 2 ml. of cell homogenate suspended in Ca-free PBS. If the interference of cell homogenate with the infection of cells by RNA occurs at the site of the cell, the efficiency of plating (eop) of infectious RNA on Ca-free PBS and cell homogenate treated cells should be different. However, no significant differences in eop were observed and one, therefore, can assume that the cell homogenate does not affect the susceptibility of the MKTC's.

The cell homogenate might be blocking the receptor sites of the facilitator and make it, therefore, impossible for the infectious RNA to adsorb to the facilitator. According to this idea, adsorption to large amounts of facilitator should remove the inhibiting capacity of cell homogenate. To test this hypothesis, 2.5 per cent (w/v) of kaolin (ten times more than used for facilitation) was added to the cell homogenate. After 30 minutes incubation at room temperature, the kaolin was removed by centrifugation. Part of the supernatant was saved to test its inhibitory effect and part of it was treated a second time the same way with fresh kaolin. Table 7 shows the effect of treating the cell harvest with kaolin prior to its interaction with infectious RNA. The untreated cell homogenate (control 2) inhibited the RNA as described above. However, once treated with kaolin, the inhibitory effect was removed to a certain extent, but still a slight depression was observed, comparing the 15 and 6 plaques obtained in the presence of kaolin treated harvest with the 31 and 14 plaques obtained with RNA without any inhibitor (control 1). When treated twice with kaolin, there was no inhibition of the RNA; the same plaque numbers were obtained as in control 1. It appears that the inhibitory effect described in this paper is related to the adsorption of the infectious RNA onto the facilitator.

Comparison of Infectious RNA Obtained by the 7 per cent and 50 per cent Phenol Methods

We have shown that cell homogenate, a number of protein containing compounds, as well as RNA and DNA inhibit the infection of MKTC's with polio RNA. Preparing the RNA of poliovirus with the 50 per cent phenol method accumulates the proteinaceous material of crude virus stocks in the phenol layer, which is discarded. The 7 per cent phenol treatment of poliovirus, which also yields RNA, does not remove cellular proteins from the virus suspension. Based on results described above, one would, therefore, expect an infectious RNA prepared with 7 per cent phenol to be less infective than one prepared with the 50 per cent method, at least when the infectivity of the undiluted RNA preparations are compared.

A crude virus stock obtained by harvesting cells 4 hours after infection was treated with 7 per cent phenol. From one part of the resulting infectious RNA, the infectivity was measured by the plaque assay on MKTC's. Another aliquot of the RNA solution was further treated with an equal volume of water saturated phenol (50 per cent phenol method) to extract the proteinaceous material. The RNA solutions were obtained from the same stock, by 7 per cent and 50 per cent phenol treatment in series. These preparations were inoculated undiluted and in ten, one hundred, and one thousand-fold dilutions. The results of a typical experiment are listed in Table 8. The 7 per cent preparation had an increased infectivity

when it was diluted ten-fold, hence was inhibited in its concentrated form. The RNA prepared with the 50 per cent method was not inhibited in its concentrated form, however its ten-fold dilution was equally infective. This phenomenon is routinely observed when RNA is facilitated with kaolin or a similar facilitator. At lower dilution steps, the plaque numbers obtained by the two differently prepared RNA's are decreasing, but not significantly different. At higher dilutions, the plaque numbers do not correspond with the dilution factor. This is another feature of infectious RNA facilitated with kaolin or similar compounds. Concluding from these results, it appears that neither RNA preparation is superior, however RNA from the 7 per cent method is inhibited when undiluted.

Table 8. Comparison of 7% and 50% Phenol Method for Obtaining Infectious RNA

Method	Arithmetic mean of the number of RNA plaques per petri at the concentration of:			
	1:1	1:10	1:100	1:1000
7%	32	ca. 100	35	7
50%	ca. 100	ca. 100	32	7

Discussion

We have demonstrated that cell homogenate as well as non-specific protein containing compounds and NA's inhibit the infectivity of polio RNA when kaolin is used as facilitator. The inhibition is reversible when the inhibitor is diluted out, and additional facilitator is added. The inhibition takes place at the receptor site of the facilitator. The facilitator is the catalyst for the infection of the cell with RNA. Cell homogenate and other substances presumably poison this catalyst probably by loading its surface and not leaving it active enough to assist the RNA in entering the cells. When RNA is prepared from high titered virus stocks the inhibitory effect of the cell debris will not come into effect because the RNA will mostly be used in a diluted form. However if RNA is prepared from low titered virus stocks, and one has to use it undiluted, one has to expect some inhibition. In this case, the 50 per cent phenol extraction is preferable.

Comparing the inhibition of infectious RNA by cell homogenates with the inactivation by RNAase, one can conclude that the MKTC cells do not have any or very little RNAase-like activity. At least under the conditions tested (37° C for 30 minutes), the cell homogenate does not destroy any measurable amount of infectious RNA. It is therefore very unlikely that the cellular RNAase inactivates the infectious RNA at the cell surface during the adsorption period. However this does not mean

that cellular RNAase might not be responsible for the low efficiency of plating of infectious RNA; but the interaction of the enzyme must take place over a longer time interval, presumably inside the cell.

The quantitative determination of the infectivity of a RNA solution is not without difficulties. The relationship of infectivity obtained as plaque numbers with the concentration of the inoculum is not proportional. To obtain most reliable results one should always plate three or more concentrations of the RNA solution. From the plaque number obtained at each dilution, one can ascertain whether the concentrated inoculum is inhibited and which could therefore give the impression of low infectivity.

The mechanism of infecting MKTC cells with infectious RNA and the exact infectivity titration still raise some questions, and it will be our future concern to investigate them.

Summary

Monkey kidney tissue culture (MKTC) cell homogenate depressed the infectivity of poliovirus RNA. This depression, however, was not due to RNAase activity of the cell homogenate, since after the depressed RNA solution was diluted and fresh facilitator was added, the infectivity was restored. Yeastolate, egg white, casein hydrolysate, and commercial RNA and DNA were found to have the same inhibitory effect on infectious RNA. The inhibitors destroyed the facilitating capacity of the facilitator. They could be easily removed by adsorbing them to large quantities of facilitator. The 7 per cent phenol treatment and the 50 per cent phenol extraction of poliovirus to obtain infectious RNA were compared. If the resulting RNA solution was inoculated undiluted, the 50 per cent method was superior because this method removed the proteinaceous material and hence part of the inhibitors in the crude virus suspension.

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