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EFFECT OF DIETHYLAMINOETHYL DEXTRANE ON THE ANTIVIRAL ACTION OF A COMPLEX OF POLYINOCYNIC AND POLYCYTIDYLIC ACIDS IN TISSUE CULTURE

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EFFECT OF DIETHYLAMINOETHYL DEXTRANE ON THE ANTIVIRAL ACTION OF A COMPLEX OF POLYINOCYNIC AND POLYCYTIDYLIC ACIDS IN TISSUE CULTURE

[Article by A. S. Novokhatskiy and F. I. Yershov, of the D. I. Ivanovskiy Institute of Virology, USSR Academy of Medical Sciences; Voprosy Virusologii (Problems of Virology), 17 March 1972, pp 312-317; submitted 12 April 1972]

Using a model of first-trypainized fibroblasts of chicken embryos and RNA-containing virus s of vesicular stomatitis and Venezuelan equine encephalomyelitis, the authors studied the conditions of the stimulating effect of a polycation of diethylaminoethyl dextrane (DEAED) on the antiviral activity of a complex of synthetic polynucleotides, namely polyinocynic and polycytidylic acids. The degree of induction of interferon-formation and suppression of virus reproduction was found to depend on the relative size of the doses of polycation and of the polynucleotide complex, as well as on the method of processing the cells and the time of their contact with the preparations. With a single treatment of the cells, using 10-20 micrograms/ml of polynucleotides per ml, and 40 - 100 micrograms of DEAED per ml, for the duration of 1 hr, suppression of reproduction of the viruses amounted to 5-6 log BSU/ml. Titers of the forming interferon did not rise above 32 BPU_{50}/ml. The DEAED evidently favored reinforcement of the complex of polynucleotides on the surfaces of the cells.

A good deal of research devoted to the study of preparations able to stimulate the formation of interferon has shown that the greatest antiviral activity is possessed by bifilar complexes of the synthetic po_ynu-

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cleotides, particularly a complex of polyinocynic and polycytidylic acids [3-5, 7, 8, 11, 13]. The antiviral activity of complexes of synthetic polynucleotides in a tissue culture is increased considerably by the presence of polycations [2, 12], which, it is presumed, favor the penetration of the polynucleotides into the cell, and protect them from degradation under the action of ribonuclease. Treatment with diethylaminoethyl dextrane (DEAED) in some conditions increases the interferonic activity of the acids when an experimental animal is injected with the preparation [10]. The present report is a presentation of the results of a study made of the conditions which will guarantee optimal effect of the DEAED polycation on the antiviral activity of polyinocynic and polycytidylic acids in a chicken embryo culture.

Material and methods.

Viruses: The viruses of vesicular stomatitis and Venezuelan equine encephalomyelitis were used, having been put through 16 and 34 passages on chicken embryo culture, respectively.

Cells. Chicken embryo fibroblasts, prepared in the usual manner, were used. Infectious activity of the viruses was determined by the plaque method, with agar. Interferon activity was studied by suppression of plaque formation by a test virus (stomatitis), and expressed in plaque-suppressive units (BFU_{50}).

Study of the antiviral action of the acids was conducted by standard methods in a CO_2 (3%) atmosphere. The following method of treating the cells was used. A 48-hour culture of chicken-embryo cells was washed with

Hanks' solution; the cells were then washed for ½ hr. with medium No. 199, containing an appropriate amount of the substance under study. Upon completion of the contact period, the culture liquid was drawn off and the cells were washed twice in Hanks' solution, and the appropriate amount of medium No. 199 with 2% beef serum was put in the test flasks. The induction period amounted to 18-20 hours, at the end of which time the culture liquid was collected and heated at 60° for 1 hr, for subsequent determination of interferon activity. The cell culture was washed with Hanks' solution, then infected with a virus (multiplicity of infection, 5-10 BSU per cell). Following contact with the virus (40 min. at 37°) the cells were again washed twice with Hanks' solution and covered with the accumulation medium (medium No. 199 with 2% heated beef serum). The virus samples were removed 24 hours following infection, each experimental point being obtained with the study of not less than three parallel samples.

Used in the research were the following: a bifilar complex of polycytidylic and polyinocynic acids, "Calbiochem"; DEAED, "Pharmacia"; pancreatic crystalline ribonuclease obtained from the Leningrad Meat Products Plant.

Results. Relationship of inhibiting action of polynucleic acids and concentration of diethylaminoethyl dextrane (DEAED). Cell cultures were treated with appropriate amounts of DEAED during the course of an hour, then the culture liquid, containing the preparation, was removed and the monolayer washed with Hanks' solution, and covered with medium No. 199, containing 10 or 20 micrograms of the polynucleic acid per ml. The control samples were covered with medium No. 199 without additives.

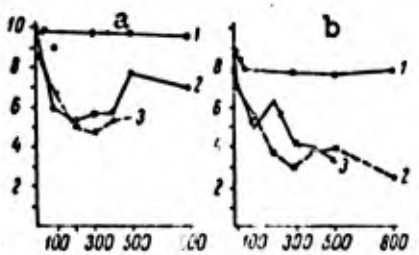

Figure 1. Effect of preliminary treatment (for 1 hr) with DEAED on the antiviral activity of complexes of polyinosinic and polycytidylic acids.

1 - processing of cells with DEAED without subsequent action of the acids; 2 and 3 - with subsequent treatment with the acids in concentrations of 10 and 20 micrograms per ml. On the y-axis are plotted virus titers, on the x-axis DEAED concentrations. Here, as in Figures 2 and 3, a denotes Venezuelan equine encephalomyelitis virus and b vesicular stomatitis virus. DEAED concentrations are given in micrograms per ml and virus titers in BSU/ml.

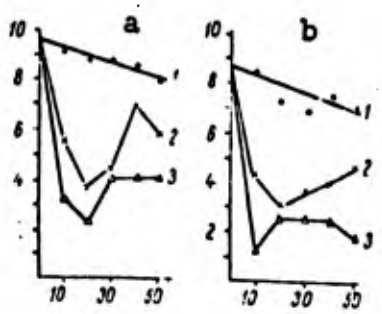

Figure 2. Effect of DEAED on the antiviral activity of polyinosinic and polycytidylic acids, during a single treatment of the cells. 1 - treatment of cells without DEAED; 2, 3 - in the presence of 40 and 100 micrograms of DEAED. Virus titers are plotted on the y-axis, acid concentrations on the x-axis.

. Subsequently the cells were treated as described above. The results are illustrated in Fig. 1. Treatment of the cells with DEAED had practically no effect on the reproduction of the virus of Venezuelan equine encephalomyelitis; it reduced the reproduction of vesicular stomatitis virus by approximately 1 log BSU/ml, which, in the absence of DEAED did not exceed 0.5-1 log BSU/ml. The antiviral effect of the acids was raised manyfold as a result of preliminary treatment of the cells with the polycation. The greatest effect of stimulation was observed with the use of 10-20 micrograms of DEAED per ml.

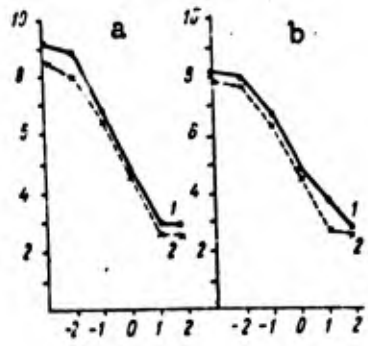

Figure 3. Effect of high concentrations of DEAED on the antiviral activity of polycytidylic and polyinocynic acids. 1 and 2, treatment of cells in the presence of 300 and 500 micrograms of DEAED per ml. Virus titers are plotted on the y-axis, acid concentrations (in log μg/ml) on the x-axis.

In this case we found a value of 5 log BSU/ml for reduction in reproduction of the model viruses.

Later on we found a more rational method of treating the cells. We applied a single treatment of medium No 199, containing the quantities

of DEAED and polynucleic acids used in the study; this made it possible to reduce the effective dosages of DEAED to 40-100 micrograms per ml.

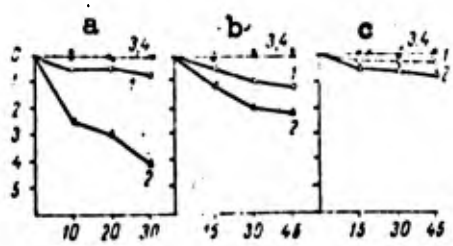

Figure 4. Efect of DEAED on the intensity of the reaction between polynucleic acids and cells under various conditions.

a - 37°. 1 - polynucleic acids without DEAED; 2 - acids with DEAED; 3, 4 - same, but with subsequent treatment with ribonuclease.

b - 4°. 1 - polynucleic acids without DEAED and without subsequent treatment of the cells with DEAED; 2 - acids without DEAED but with subsequent treatment of the cells with DEAED; 3, 4 - same, but with treatment with ribonuclease.

c - 4°. 1 - polynucleic acids without subsequent treatment of the cells with DEAED; 2 - polynucleic acids with DEAED and subsequent treatment with DEAED; 3, 4 - same but with treatment with ribonuclease. Degree of suppression of reproduction of encephalomyelitis virus is plotted on the y-axis; time of contact of virus with cells is plotted on the x-axis (in minutes).

In Fig. 2 are illustrated the results of our study of the relationship between inhibition of reproduction and dose of the polynucleic acids, with use of comparable concentrations of DEAED. In the absence of the poly-

cation, increase in the dose of the polynucleotide complex produced a linear increse in the suppression of virus reproduction, which, however, did not exceed 1-2 log BSU/ml. Addition of DEAED had this result: for 10-20 micrograms/ml, the maximal suppression of the reproduction of both viruses was achieved (reproduction reaching 5-6 log BSU/ml). Of interest was a certain reduction of antiviral activity of the polynucleotides in the case of combining equivalent or nearly equivalent quanties of the preparations under study (30-50 micrograms/ml).

The results obtained support the conclusion that increase in dose of the polycation could compensate for reduction in the dose of polynucelotide used. The data illustrated in Fig. 3 show that with use of large doses of DEAED an appreciable suppression of virus production is observed even when treating the cell layer with as little as 0.1 microgram/ml of the polynucleotide; nevertheless, the maximum of inhibition did not appear for 10-20 microgram/ml of polynucleotide, nor did it depend very substantially on the DEAED dose size.

Thus, optimal results were obtained with a single treatment of the chicken-embryo culture at 37° for 1 hour, using 10-20 micrograms of polynucleotide and 40-100 micrograms of DEAED, per milliliter.

Effect of DEAED on the stimulation of interferon-formation in a cell culture under the influence of polynucleotides. Since even a quite insignificant amount of polynucleotide, given sufficiently prolonged contact with tissue cells, may perceptibly suppress plaque formation by test viruses, we heated all samples of the culture liquid which were used to determine interferon activity, striving in this way to guard against the

effects of any possible remnants of polynucleotide; these, of course, would entirely lose any possibility of action upon being heated at 60° for the course of an hour. Interferon induced by a preparation of polynucleotide in a chicken-embryo culture, as regards resistance to heat and processing with acid pH, does not differe from ordinary chicken interferon.

Induction of Interferon-Formation in a Chicken-Embryo Culture, Produced under Various Conditions of Processing Cells with a Polynucleotide Complex

a Условия обработки клеточной культуры	Титр интерферона (в $\log_2$ $БИЭ_{50}$/мл) b
c ПолиНЦ, 1 час	
10 мкг/мл	1,8
20 мкг/мл	1,3
30 мкг/мл	1,0
40 мкг/мл	1,2
50 мкг/мл	1,9
d ПолиНЦ, 24 часа	
10 мкг/мл	1,1
20 мкг/мл	2,3
30 мкг/мл	3,4
40 мкг/мл	3,1
50 мкг/мл	3,5
e ПолиНЦ (10 мкг/мл) и ДЭАЭ-Д (100 мкг/мл), 1 час	4,1
f ПолиНЦ (1 мкг/мл) и ДЭАЭ-Д (30 мкг/мл), 24 часа	4,2
g ПолиНЦ:ДЭАЭ-Д	
2/1 1 час	2,1
1/1 1 час	3,3
1/2 1 час	4,5
1/4 1 час	4,9
1/10 1 час	4,7
h ПолиНЦ (10 мкг/мл) и ДЭАЭ-Д (100 мкг/мл)	
непрогрета интерферонсодержащая жидкость	4,3
жидкость прогрета при 60° 1 час	4,2
жидкость обработана pH 2,0	4,5

Key: a - Conditions of treating the cell culture; b - Interferon titer (in $\log_2 BPE_{50}$/ml); c - Polynucleotide complex, 1 hour, in micrograms per milliliter; d - same, but time 24 hors; e - Polynucleotide (10 micrograms per milliliter) and DEAED (100 micrograms per milliliter, 1 hour; f - Polynucleotide and DEAED, 1 and 30 micrograms per milliliter respectively, time 24 hours; g - Polynucleotide and DEAED in proportions indicated, time 1 hour; h - Polytide and DEAED, 10 micrograms per milliliter and 100 micrograms per milliliter, respectively, with three conditions specified: no heating of the interferon-containing liquid, heating at 60° for 1 hour, and liquid processed at pH 2.0.

As is evident from the preceding table, duration of contact of the preparations with the cells, and also the dose size of the polynucleotide and the DEAED had a substantial effect on the level of interferon-formation. Whereas with short-term treatment (1 hour), without the use of DEAED, it was possible to produce only insignificant amounts of interferon in the culture medium, as the contact time was increased up to 24 hours there appeared an accompanying increase in interferon titer, which was proportional to the polynucleotide dose. The presence of DEAED assured stimulation of the formation of sizeable quantities of interferon iven in the case of one-hour contact; increase in processing time made it possible to reduce the doses of preparation necessary. We derived an expression for the relationship between induction for the relationship between induction of interferon-formation and the quantitative connection between polynucleotide and DEAED. The highest interferon titers were found when the DEAED dose was not less than 4-10 times as great as the polynucleide dose.

One should emphasize that interferon titers whose formation was stimulated under particular conditions by a preparation of polynucleotides in a chicken-embryo culture, did not in our experiments exceed 5 log BPE_{50}/ml, whereas viral induction produced, in the same cell culture, the formation of 10 - 11 $\log_2 BPE_{50}$/ml.

The Effect of DEAED on the Process of Interaction between Polynucleotides and Chicken-Embryo Culture. The cells were treated once only, with 10 micrograms of polynucleotide and 50 micrograms of DEAED per milliliter. Contact of the cells with the preparations was maintained at 37° and 4°; when the latter temperature was used, the cultures were given preliminary chilling in a refrigerator for 30 minutes. We determined the dynamics of the interaction between polynucleotides and cells, varying duration of contact from 10

up to 30 minutes, at a temperature of 37°, and from 15 to 45 minutes at 4°. Some of the culture flasks, following the necessary time of contact, were washed twice in Hanks' solution, covered with medium No. 199; to the others, following washing, was added medium No. 199 with 50 micrograms of DEAED. After 20 minutes at 37°, the culture liquid in one batch or the other was removed, the cells washed and covered with nutritive medium. The cell cultures so treated were once again divided into two portions, one of which was subjected to the action of ribonuclease (0.25 μg/ml for 1 hr.) at 37°. Next the cell cultures were washed free of ribonuclease, covered with nitritive medium, and, at the end of the period of incubation infected with Venezuelan stomatitis virus.

The results of the experiment are illustrated in Fig. 4. As is clear, at 37° the presence of DEAED significantly accelerated the interaction of polynucleotide with the cells, but did not protect the preparation attached to the cells from inactivation under the influence of a considerable amount of exogenous ribonuclease (Fig. 4-a). Treatment of the cells with 50 μg of DEAED following the contact period at 37° did not substantially alter the period.

With use of a 4° temperature (Fig. 4 b and c) a more active interaction between the polynucleotide and the cells occurred without the use of DEAED. This circumstance, described previously by Tilles [12], was evidently brought about by increase in the amount of DEAED at that temperature, which rendered contact of the preparation with the cell surfaces more difficult. In all cases, the action of 0.25 mg/ml of ribonuclease, capable, in the concentration used, of destroying polynucleotides, [12], completely prevented the

formation of an antiviral protection of the cells.

Discussion. Intensification of the antiviral effect of polynucleotides, under the influence of the polycation of DEAED, proceeds in varying degrees, depending on the use of one type of cell system or another. The results we obtained made possible a justifiable determination of optimal conditions in treating single-layer chicken-embryo cultures so as to assure development of the protective effect with use of minimal doses of polynucleotides and DEAED. With a single treatment of cells during the course of 1 hour, the action of 10-20 μg/ml of polynucleotide and 50-100 μg/ml of DEAED, capable of assuring suppression of the reproduction of certain RNA-containing viruses by 5-6 log BOE/ml, is effective at least during the first 24 hours following infection.

When present in the nutrient medium in quantities nearly equivalent, DEAED and polynucleides may form conglomerates [10] whose activity is significantly reduced. In such instances, where, for the development of the antiviral effect no prolonged contact between polynucleotide and cells is required, one may employ a single application of polynucleotides and polycation. If, however, the prolonged presence of a complex of polynucleotides in the culture medium is necessary, then one should employ preliminary processing of the cells with larger doses of the polycation, or else carefully avoid a polynucleotide-DEAED ratio which is close to equivalent.

The increasing effect of DEAED on the protective function of polynucleides in the conditions of our tests was exerted during the very first stages of the reaction between the polynucleotide complex and the cells,

when properly the development of the antiviral effect can be expected only in the course of the induction period, and when treatment with ribonuclease may completely prevent its appearance. Comparison of the intensity of the interaction between polynucleotides/ and cells at 4° and 37°, and with DEAED and without it, leads to the conclusion that the polycation either reinforces the complex of polynucleotides on the cell surface, or strengthens the rise of the polynucleotide within the cell. In this connection, however, it should be taken into account that research conducted with a tagged preparation [1] has fully confirmed that the addition of 100 micrograms/ml of DEAED produces a thirty-fold increase in the quantity bound with the surfaces of the cells of human fibroblasts; only an insignificant portion of the DEAED, in fact, penetrates deeply within the cell, where it is destroyed and reutilized for synthesis of cellular nucleic acids.

A point of interest is the fact that in the majority of cases [6, 9] the antiviral effect exceeds the effect of the stimulation of interferon-formation. The results which we obtained demonstrate the presence of the existence of a link between the conditions which favor maximal induction of interferon and the development of maximal antiviral action. Presently available information does not warrant any clear decision as to whether the interferon mechanism of action, or one similar to it, is the unique element in the effect of polynucleotides/ , or simply a lateral concomitant. Possibly--and indeed most probably--it is only such a lateral concomitant: in other words, one of the component parts of the mechanism of antiviral action by a complex of polynucleotides.

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