TRANSLATION NO. 2780

DATE: 2 December 1971

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

Vop. Virusol 16:3:317-322, 1971

Acad. Sci. Pub. 576.858.25.095.6.083.35.095.18

The effect of DEAE-dextran, protamine sulfate and sulfated polysaccharides of agar on multiplication of Venezuelan equine encephalomyelitis virus in cell cultures

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The model of VEE virus and trypsinized chick embryo cells was used to study the effect of diethylaminoethyl-dextran (DEAE-D), agar polysaccharides (APS) and protamine sulfate (PS) on the pattern of virus-cell interaction. The presence in the growth medium of DEAE-D and PS inhibited virus production. The inhibiting effect of DEAE-D was manifested in the first stages of virus-cell interaction and was more marked with increasing pH. Sulfated polyanions of agar inhibited virus multiplication affecting later stages of reproduction. Lowering of pH of the growth medium activated the inhibiting effect of polysaccharides.

Many works have been published recently which have been devoted to a study of polycations and polyanions influence on the process of reproduction of arboviruses in the culture fabric (9, 13 - 15). In several works, reports were made on the stimulating influence of DEAE-D on the productivity of cells (4). Taking into account that DEAE-D and PS specifically improve the formation of platelets of these arboviruses (8, 12 13), we tried to make a more detailed study of the influence of these substances on the reproduction of VEE viruses in the cultures of trypsinized chick embryo cells. A basic indicator of productivity of the cells was the growth in the culture medium of the infectious virus, the titers of which were determined by the method of platelets under agar.

Material and Methods

Viruses. The VEE virus was received in 1944 from the collection of the Rockefeller Institute. In connection with the fact that the parent strain of VEE viruses gave varying sizes of platelets, we tried to separate genetically, a pure line of viruses. After a five-fold seried passivation from one platelet we received a stable fine-plated variant, which at the present time has gone through 28 passages in trypsinized chick embryo cells.

Semlik forest viruses (SFV), Sindbis (SV) and viruses of vezicular stomatite of the Indiana strain (VSV) were received from the virus museum of the Institute of virology of the U.S.S.R. Academy of Medical Sciences and these went through several passages in the trypsinized chick embryo cells. These cells were prepared from 10 - 11 day chick embryos by the usual methods (2).

The infectious activity of the virus was determined when necessary with the use of support methods in a thermostat, biosphere of which contained 3% CO_2 (3, 5).

APS was received by freezing out 2% of Lifko agar, as described by Takemoto and Liebhaver (16).

DEAE-D (Pharmacia, Uppsala, Sweden) was applied as an autoclaver for 10 minutes in a sterile 1% solution.

PS (Spofa, Prague, C.S.S.R.) was applied in a 1% sterile solution.

Agarose (Industrie, Biologique Francaise) was used in a series of cases for the formation of a feeding cover with a final concentration of 1%.

Results. The action of DEAE-D and PS on the platelet formations of the arbovirus group A: The VEE formed, under an agar covering which did not contain polycations, round platelets with even edges 0,5 - 1,5 mm. in size. DEAE-D and PS upon addition to the agar covering caused an increase of 4 - 6 times in the size of the VEE platelets, the Sindbis and Semlik forest platelets also. The concentration of DEAE-D in the presence of which was observed the greatest increase in the size of the platelets consisted of 800 mkg/ml. The addition in the quantity of more than 2000 mkg/ml. led to the death of the cell culture in 48 to 72 hours from the toxic influence of the polycations. The optimal concentration of PS was 400 - 600 mkg/ml; the toxic effect appeared in 48 - 72 hours of incubation with the contents of PS being 1000 mkg/ml and more. An insignificant increase in the number of platelets received in the presence of DEAE-D and PS, followed, in our opinion, is related to the extremely small platelets in the absence of DEAE-D and PS and in the usual conditions it is not counted. In the composition of the covering used, the addition of DEAE-D in the quantity of 100 mkg/ml did not exert influence on the number of VSV platelets of the Indiana strain.

In that case where for the preparation of the covering, agarose was used (final concentration 1%), which did not contain sulfated polyanions, the VEE platelets in the absence of DEAE-D were identical in size to the platelets received in the usual agar covering in the presence of 800 mkg/ml of DEAE-D.

The influence of DEAE-D on the size of the platelets of the VEE variants differing in their level of thermoresistence: During study of the inactivation dynamics of VEE at various temperatures, variants were received which differed in their level of thermoresistence (6).

In sketch 1 is shown the inactivation dynamics t^r, t^s, and t^{sl} of the VEE virus at 54 degrees. If the t^r strain inactivated at 1 lg PFU/ml in 45 minutes, then the loss of 1 lg infectious activity of the t^s strain took place in 10 minutes, which was sufficient for an almost whole inactivation of the t^{sl} variant. In sketch 2 is shown the graph summarizing the percent of the platelet sizes of the various variants of VEE received at 36 degrees under an agar covering, either containing or not containing 800 mkg/ml of DEAE-D.

In the absence of DEAE-D a reliable difference in the platelet sizes of the t^r and t^s variants was not discovered. The addition of 800 mkg/ml of DEAE-D exerted varying influence on the formation of the platelets of the VEE variants. The most thermoresistant of them caused a relatively small increase in the platelet size, when the t^s and particularly the t^{sl} formed platelets of particularly large size.

The influence of various concentrations of DEAE-D and PS on the multiplication of VEE virus: Having formed a monolayer of chick embryo cells we washed them twice with Henkes solution and infected them for 40 minutes of contact at 36 degrees with VEE with an infection multiplicity

of 5PFU/cell. The infectious activity of the virus was determined in samples taken 24 hours after infection. The addition to the growth medium (medium No. 199 with 2% bovine serum) of PS, from 15 to 300 mgk/ml (sketch 3), led to a progressive fall in the VEE harvest, the logarithm of infectious activity of which was within studied limits almost the opposite in proportion to the logarithm of the PS concentration.

The DEAE-D added to the growth medium in a quantity of from 50 to 2000 mgk/ml, likewise exerted inhibiting influence proportional to the logarithm of its concentration, but the suppression of the multiplication of the virus was expressed more weakly (sketch 4). With the goal of cleaifying the action of the components of the heated bovine serum on the inhibiting action of the polycations, a series of experiments were done, in which we used as a growth medium, Earle's solution with 0,22% of sodium bicarbonate.

In the control experiments, the virus accumulated from 1 - $5 \cdot 10^8$ PFU/ml. Using as a medium of growth the Earles solution, the 300 mkg/ml, of PS inhibited the virus multiplication tc the same degree as when we used medium No. 199 with 2% bovine serum. In the case of addition of DEAE-D to Earle's solution with 0,22% of sodium bicarbonate, the virus titers were in all cases from 2 - 3 lg PFU/ml lower than in the medium No. 199 with 2% bovine serum containing the same quantity of polycation.

Inactivation of VEE at various temperatures in the presence of DEAE-D and PS; In a special series of experiments with the inactivation of VEE at 4, 37 and 54 degrees, it was proven that there was no spontaneous influence of PS and DEAE-D on the virions.

During 24 hours at 4 degrees the PS in a concentration of 2000 mkg/ml did not cause perceptible decrease in the infectiousness of the virus. The inactivation of VEE at 50 degrees in the presence of 1000 mkg/ml of DEAE-D proceded identically with the control.

The influence of treatment of the cells with DEAE-D and APS for various periods of infection: In table 1 are the results of the determined influence of the cells pretreated with DEAE-D and APS on the production of VEE, and likewise results of the study of the action of these substances on the various stages of interaction of the virus with the cells. The results introduced are average indicators from 3 - 5 experiments. The greatest degree of inhibition of the virus multiplication in the presence of cells treated with DEAE-D (1000 mkh/ml) was observed in the case of 4-hour contact of the polycation with the cells: 2 hours before infection of the cells and 2 hours after. It is necessary to note that treatment of the cells with DEAE-D (1000 mkg/ml) for 4 hours begun 2 hours after infection of the cells, in fact, did not lower the virus harvest. Sulfated polyanions of agar in a series of experiments are shown in table 1; they were applied as a polyliquid agar covering. In the control series of experiments, we used the same prescription as a covering with the addition of DEAE-D (800 mkg/ml). The most expressed suppression of virus multiplication was noted on contact with the polyliquid agar covering which did not contain DEAE-D, from the third through the sixth hour after infection.

The influence of pH and infection multiplicity: In tables 2 and 3

are shown the results of determination of the influence on DEAE-D and APS inhibiting activity exerted by such cultivation conditions as pH, aeration with 3% CO_2 and infection multiplicity. The 2 multiplications of infection - 5 and 0,0005 PFU/cell - were used. In the growth medium (medium No. 199 with 2% bovine serum) there was present during the whole incubation period 10% extract of APS or 1000 mkg/ml of DEAE-D. The value of pH is established by the changes of color of the phenol red indicator, adding the indispensible quantity of 7,5% solution of sodium bicarbonate or skipping the CO2. In the presence of 10% APS the virus harvest depended on the infection multiplitiicy if the pH medium of growth was equal to 7,0. With a higher value of pH in the atmosphere containing CO_2, the degree of inhibition did not depend on the infection multiplicity. In all cases in the presence of APS the virus titers were by 1 - 2 lg PFU/ml lower than in the control. The inhibiting action of DEAE-D did not depend on the infection multiplicity, but was more expressed upon increasing the pH to 7,7 in the atmosphere containing 3% CO_2.

Discussion. The size and character of the arbovirus platelets received under an agar covering depends on the action of many factors: the incubation temperature, pH and the composition of the feeding covering, the type of cells and the biological properties of the viruses. Other conditions being equal, the variations in the structural organization and properties of the proteins of the virion casing exert specific influence on the size of the platelets. This clearly is supported by the formation of VEE variants inthe presence of DEAE-D, of APS neutralizing inhibition activity, of platelets of varying size, inasmuch as the resistance of the described variants is determined by the peculiarities of their protein casings (6).

Polycations and polyanions exert an expressed influence on the interrelation of cells and virus and in part on the process of platelet formation. During the simultaneous presence of these substances, capble of actively interacting with the surface formations of the cells (11) and viruses (9,13), their influence to a significant degree is neutralized; it is logical to explain the stimulation of platelet formation of the viruses under study in the presense of DEAE-D added to agar coverings by the latter fact. However, added separately, they can suppress the production of viruses in the cell culture, although this action to a large degree depends on the cultivation conditions determined by the phico-chemical properties of the feeding medium. The high-molecular components of bovine serum decreases the effect of the DEAE-D and PS action. The inhibiting action of APS on the multiplication of arboviruses is also suppressed by the addition of bovine serum (1, 13).

The reaction level of the medium, established by the addition of a varying quantity of sodium bicarbonate or aeration with a mixture of air with a prescribed concertration of CO_2, exerts a contradictory action on polycations and polyanions. The inhibiting effect of DEAE-D is activated during increase of the pH of the medium, since APS is more active during decrease of pH.

Suppression of the reproduction of VEE viruses had various mechanisms during the action of DEAE-D and APS. Addition of polycations did not inactivize the virus particles, but its influence appeared most active in the first stages of interaction of the virus with the cells. In several virus systems - cell systems, this influence could ease the

process of virus adsorbtion and its penetration into the cells and, apparently, as a result of increase in the number of infected cells, heighten the virus harvest (4). The virus particles are inactivated by APS besides which they can penetrate inside the cell accumulating in the cytoplasm (17), to suppress the virus reproduction, exerting influence on the later stages of their multiplication. In table 4 is a brief summary of the various mechanisms of action of DEAE-D and APS.

A more significant suppression of VEE virus multiplication takes place under the influence of APS at pH 7,0, and a small infection multiplicity (see table 2) is, probably, the result of heightened inactivating action of the polyanions. This permits drawing a parallel with the formation mechanism of the d-sign, studied on the model of the poliomyelitis virus, (1). To what degree is is possible to calculate the receipt of DEAE-D, and of VEE variants sensitive at high pH in the absence of APS, is the subject pointing to further research.

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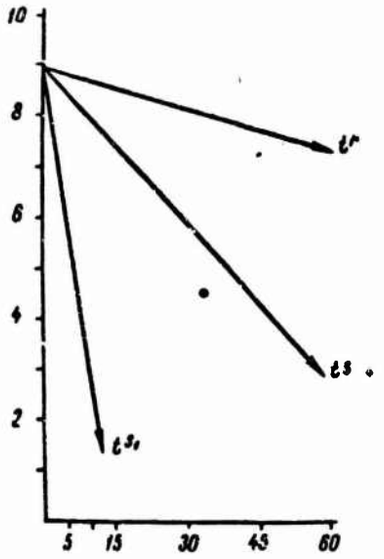
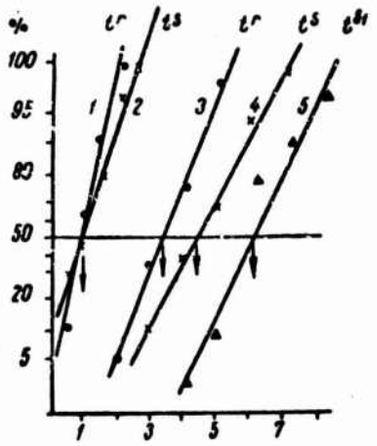
1.



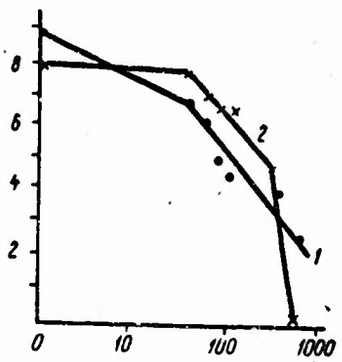


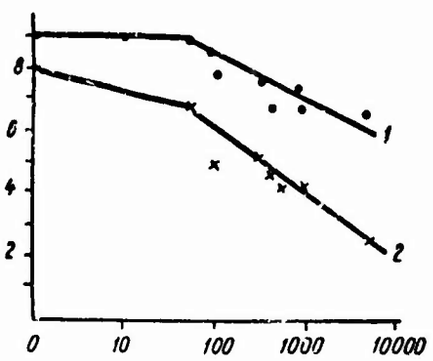
2.

Sketch 1. Kinetics of inactivation of the VEE virus variants at 54 degrees. Abscissa - heating time (in minutes). Ordinate - infection titer of the viruses in relative units.

Sketch 2. Platelet size of the VEE virus variants, differing in levels of thermoresistence, in the absence of DEAE-D (1,2), and in the presence of DEAE-D (800 mkg/ml) as a covering (3,4,5). Abscissa - platelet size (in mm.). Ordinate - relative quantity of platelets of a given size (in %).

3





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Sketch 3. Accumulation level of VEE virus in the presence of various quantities of PS in No. 199 medium with 2% bovine serum (1) and in Earle's solution with 0,22% sodium bicarbonate (2). Abscissa - quantity of PS (in mkg/ml). Ordinate - lg PFU/ml.

Sketch 4. Accumulation level of VEE viruses in the presence of various quantities of DEAE-D in No. 199 medium with 2% bovine serum (1) and in Earle's solution with 0,22% sodium bicarbonate (2). Abscissa - quantity of DEAE (in mkg/ml). Ordinate - lg PFU/ml.

Table 1. Inhibition of VEE virus multiplication by DEAE-D and APS introduced in varying stages of interaction of the virus with the cells

Conditions of cell treatment	Degree of virus inhibition (in lg PFU/ml)	
	In presence of DEAE-D (1000 mkg/ml)	In treatment with poly-liquid agar covering without DEAE-D
2 hours before infection	0,2	0,3
At the moment of infection	-	0
2 hours after infection	0,2	0,8
2 hours before and 2 hours after infection	1,0	-
4 hours in 2 hours after infection	0,1	3,1

Table 2. Influence of cultivation conditions on the VEE virus multiplication in the presence of agar polysaccharides

Cultivation conditions	Infection multiplicity (PFU/cell)	Virus harvest (in lg PFU/ml)	
		No. 199 medium with 2% bovine serum	No. 199 medium with 2% bovine serum and 10% APS
In atmosphere of 3% CO_2, pH 7,8	5	10,0	7,8
	0,0005	9,3	7,7
Without aeration, pH 7,0	5	9,8	8,0
	0,0005	9,7	6,1

Table 3. The production of VEE virus in the presence of DEAE-D (1000mkg/ml) in various conditions

Cultivation conditions	Infection multiplicity (PFU/cell)	Virus harvest (in lg PFU/ml)	
		No. 199 medium with 2% bovine serum	No. 199 medium with 2% bovine serum and DEAE-D (1000mkg/ml)
Aeration of 3% CO_2, pH 7,7	5	9,0	7,0
	0,0005	8,9	7,0
Without aeration, pH 7,0	5	8,8	7,7
	0,0005	8,6	7,7

Table 4. Various mechanisms of action of DEAE-D and APS

DEAE-D - polycations	APS - polyanions
Do not exert influence on virions	Inactivate virions
Influence first stages of interaction of virus with cells	Influence subsequent stages of interaction of virus with cells, accumulating in cytoplasm
Activate during high pH	Activate during low pH