Plaque Enhancement of Enteroviruses by Magnesium Chloride, Cysteine, and Pancreatin

CRAIG WALLIS, FRED MORALES, JOYCELYN POWELL, AND JOSEPH L. MELNICK

*World Health Organization International Reference Centre for Enteroviruses, Department of Virology
and Epidemiology, Baylor University College of Medicine, Houston, Texas*

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ABSTRACT

WALLIS, CRAIG (Baylor University College of Medicine, Houston, Tex.), FRED MORALES, JOYCELYN POWELL, AND JOSEPH L. MELNICK. Plaque enhancement of enteroviruses by magnesium chloride, cysteine, and pancreatin. *J. Bacteriol.* **91**: 1932-1935. 1966.—Plaque formation of 21 echoviruses (types 1-6, 9, 13, 15-19, 23-26, 29-32) and 8 coxsackieviruses (B1-6, A7, and A9) was enhanced by increased concentrations of $MgCl_2$, L-cysteine, and pancreatin in agar overlay medium. In most cases, cationic and anionic polymers (diethylaminoethyl dextran, dextran sulfate, or protamine sulfate) were ineffective. All strains of poliovirus and group B coxsackieviruses were enhanced under agar by $MgCl_2$. Five of the eight coxsackieviruses tested were also enhanced by cysteine or pancreatin. Certain enteroviruses, which have been difficult to assay by plaque method, can now be quantified effectively by incorporation of additives such as $MgCl_2$, cysteine, or pancreatin into the overlay medium.

Since the initial observation by Takemori and Nomura (11) that agar polysaccharides may contain inhibitors of poliovirus, replication of a number of viruses has been shown to be inhibited under agar (1-7, 9, 10, 13, 14, 18). In all the cases cited above, Noble agar was used for overlays. We have previously shown that monkey kidney cells do not do as well under Noble agar as they do under bacteriological agar (Difco), and that Noble agar may inhibit the growth of certain viruses (18). These findings have recently been confirmed by Nagai and Hammon (8).

Recently, we observed that type 4 echovirus produced earlier and larger plaques when additional cysteine was incorporated into the medium (17), and that poliovirus and reovirus plaque formation is enhanced, respectively, by $MgCl_2$ (15) and pancreatin (Wallis, Melnick, and Rapp, *in preparation*). This present study is concerned with the effects of $MgCl_2$, cysteine, and pancreatin on a large number of enteroviruses. These enhancing agents were compared with the anionic and cationic polymers used by the workers referred to in the above paragraph.

MATERIALS AND METHODS

Monkey kidney cells. Kidneys from immature rhesus monkeys were trypsinized and grown in

Melnick's medium A containing 0.2 mM $AlCl_3$ to suppress adventitious agents (16).

Viruses. Virulent strains of poliovirus were plaque-purified lines of type 1, Mahoney; type 2, MEF₁; and type 3, Saukett. Attenuated strains were Sabin's plaque-purified lines as used in the oral poliovaccine (type 1, LSc; type 2, P712; and type 3, Leon). Other enteroviruses that grow in monkey kidney cells which were also included were 27 prototype echoviruses (types 1-7, 9, 11-19, 22-27, 29-32) and 8 prototype coxsackieviruses (types A7, A9, B1-6). All virus stocks were grown in monkey kidney cells maintained with Melnick's medium B, and representative harvests containing high plaque-forming unit (PFU) titers were used in this study. All viruses were identified with reference typing antisera, many of which were obtained from the Research Reference Reagents Branch, NIAID; other reference sera were those of the World Health Organization International Reference Centre for Enteroviruses.

Virus assays. Viruses were titrated in monkey kidney bottle cultures by measuring PFU. Overlay medium consisted of Earle's salt solution, 0.4% $NaHCO_3$, 1:60,000 neutral red, 0.1% skim milk, 1.5% agar (Difco; 18), plus additives in concentrations described below: (i) 25 mM $MgCl_2$ (15); (ii) 100 $\mu g/ml$ of diethylaminoethyl (DEAE) dextran (13; Pharmacia, Uppsala, Sweden); (iii) 100 $\mu g/ml$ of sodium dextran sulfate (13; Nutritional Biochemicals Corp., Cleveland, Ohio); (iv) 400 $\mu g/ml$ of protamine sulfate (14; Salmine, Nutritional Biochemicals Corp.);

(v) 1 mM L-cysteine (17; a 100 mM stock of L-cysteine was made by solubilizing cysteine hydrochloride monohydrate in distilled water; NaOH was used to neutralize the solution; this stock was diluted 100-fold in overlay medium); (vi) 1:60 pancreatin (Oxoid, Colab Laboratories, Inc., Chicago Heights, Ill.; Wallis, Melnick, and Rapp, *in preparation*; buffered pancreatin tablets were dissolved in 50 ml of distilled water, as described by the manufacturer, and filtered through an 0.22- μ Millipore filter; This solution was frozen as undiluted stock until used, at which time it was diluted 1:60 in agar overlay medium).

The results in each table are the averages of three different tests with each virus. At least four 1-oz bottles were used with each virus for each test. Plaque counts and sizes were determined on the day discrete plaques formed in control cultures.

RESULTS

Plaque enhancement of poliovirus. Three strains of attenuated and virulent polioviruses, representing the three immunological types, were inoculated in 0.1-ml amounts into drained monkey kidney cultures in flat 1-oz bottles, with the use of an input of 20 to 40 PFU per culture. After 1-hr adsorption at 37 C, representative cultures were overlaid with agar medium with and without additives shown in Table 1. In each case the $MgCl_2$ overlay resulted in increased plaque counts and sizes as compared with the cultures with the usual overlay. DEAE dextran increased plaque counts of type 1 attenuated virus, but dextran sulfate inhibited the plaque formation of this virus. Takemoto and Kirschstein (12) have already reported such findings with Noble agar. With Difco agar we found that DEAE dextran and dextran sulfate had no influence on the type 2 and type 3 polioviruses. Similarly, protamine sulfate, cysteine, and pancreatin afforded no advantage.

Plaque enhancement of echoviruses. Prototype

echoviruses were inoculated as described above. As shown in Table 2, of the 27 viruses tested, only 5 were not affected by the additives (types 7, 11, 12, 14, and 27). Although the plaque count of type 12 was not increased in the presence of $MgCl_2$, nor of type 14 by cysteine, the plaques were two to three times the size of those in control cultures in additive-free overlays.

Six echoviruses were inhibited by one or more of the additives: type 1 by protamine, type 9 by dextran sulfate, type 16 by $MgCl_2$, type 22 by cysteine, type 23 by cysteine and pancreatin, and type 32 by $MgCl_2$ and dextran sulfate.

$MgCl_2$ enhanced the plaque counts of 11 echoviruses (types 1, 2, 3, 5, 6, 9, 13, 15, 19, 23, and 25). DEAE dextran enhanced only echovirus 17; dextran sulfate, types 6, 17, 23, 29, and 31; protamine sulfate, types 3, 5, 6, 13, 17, 19, 22, and 25; cysteine, types 4, 9, 13, 15, 16, 17, 18, 19, 24, 25, 26, 29, 30, 31, and 32; and pancreatin, types 15, 24, 25, 26, 30, and 32.

Plaque enhancement of coxsackieviruses. Prototype coxsackieviruses were tested as described above. The results of the three experiments are shown in Table 3. All group B coxsackieviruses tested were enhanced by $MgCl_2$ in the overlay medium. Plaques of A9 were also enhanced but not those of coxsackievirus A7. B1 and B2 plaques were inhibited by dextran sulfate. DEAE dextran enhanced the plaque counts of B4 and B5; dextran sulfate, B5 and A9; protamine sulfate, B4 and B5; cysteine, B3, B4, B5, B6, and A7; and pancreatin, B3 and A7.

DISCUSSION

In this study, $MgCl_2$, L-cysteine, and a proteolytic enzyme (pancreatin) have been compared with anionic and cationic polymers, for their effects on the plaque formation of enteroviruses.

TABLE 1. *Plaque enhancement of polioviruses*

Type and strain	Plaque size of control cultures	Additives in agar overlay medium*					
		$MgCl_2$, 25 mM	DEAE dextran, 100 μ g/ml	Na dextran sulfate, 100 μ g/ml	Protamine sulfate, 400 μ g/ml	Cysteine, 1 mM	Pancreatin, 1:60
	mm						
I. LSc.....	2.0	2 (2)	2 (2)	-3 (-2)	0	0	0
I. Mahoney.....	3.0	2 (2)	0	0	0	0	0
II. P712.....	2.0	3 (2)	0	0	0	0	0
II. MEF ₁	3.0	2 (2)	0	0	0	0	0
III. Leon.....	1.0	3 (3)	0	0	0	0	0
III. Saukett.....	2.0	3 (3)	0	0	0	0	0

* To simplify the evaluation of this and subsequent tables, the results of plaque counts and sizes have been scored as the multiplicity of increased counts based on an average of four cultures each; 2 (2) indicates the plaque count was two times that of the additive-free control cultures and the plaque size was two times greater; -3 (-2) indicates the plaque count was one-third and the plaque size one-half that of the control; 0 indicates plaque count and size same as controls.

TABLE 2. *Plaque enhancement of echoviruses**

Echovirus type	Plaque size of control cultures	Additives in agar overlay medium					
		MgCl ₂ , 25 mM	DEAE dextran, 100 µg/ml	Na dextran sulfate, 100 µg/ml	Protamine sulfate, 400 µg/ml	Cysteine, 1 mM	Pancreatin, 1:60
	<i>mm</i>						
1	2.0	3 (3)	0	0	-2 (0)	0	0
2	1.0	2 (2)	0	0	0	0	0
3	0.5	2 (2)	0	0	2 (4)	0	0
4	1.0	0	0	0	0	8 (3)	0
5	0.5	2 (2)	0	0	2 (4)	0	0
6	0.5	3 (3)	0	2 (2)	2 (4)	0	0
7	3.0	0	0	0	0	0	0
9	0.5	2 (2)	0	-2 (-2)	0	5 (2)	0
11	0.5	0	0	0	0	0	0
12	2.0	0 (2)	0	0	0	0	0
13	0.5	2	0	0	3 (3)	3 (2)	0
14	1.0	0	0	0	0	0 (3)	0
15	0.5	3 (0)	0	0	0	5 (0)	3 (0)
16	0.5	-2 (0)	0	0	0	2 (2)	0
17	0.5	0	4 (0)	3 (0)	3 (6)	5 (0)	0
18	0.5	0	0	0	0	3 (3)	0
19	0.5	2 (2)	0	0	2 (4)	3 (3)	0
22	0.5	0	0	0	2 (2)	-5 (0)	0
23	0.5	2 (2)	0	0	0	-2 (0)	-3 (0)
24	2.0	0	0	0	0	3 (3)	2 (2)
25	0.5	3 (3)	0	0	3 (5)	3 (0)	4 (2)
26	0.5	0	0	0	0	2 (2)	3 (3)
27	2.0	0	0	0	0	0	0
29	2.0	0	0	2 (2)	0	3 (3)	0
30	1.0	0	0	0	0	3 (2)	2 (2)
31	1.0	0	0	5 (2)	0	10 (3)	0
32	0.5	-4 (-2)	0	-5 (-2)	0	8 (2)	10 (3)

* See footnote to Table 1.

TABLE 3. *Plaque enhancement of coxsackieviruses**

Coxsackievirus type	Plaque size of control cultures	Additives in agar overlay medium					
		MgCl ₂ , 25 mM	DEAE dextran, 100 µg/ml	Na dextran sulfate, 100 µg/ml	Protamine sulfate, 400 µg/ml	Cysteine, 1 mM	Pancreatin, 1:60
	<i>mm</i>						
B1	0.5	3 (2)	0	-3 (0)	0 (3)	0	0
B2	0.5	2 (2)	0	-3 (0)	0 (3)	0	0
B3	2.0	2 (2)	0	0	0	3 (2)	3 (3)
B4	0.5	3 (3)	2 (2)	0	2 (3)	2 (2)	0
B5	0.5	3 (3)	3 (3)	3 (3)	4 (3)	4 (2)	0
B6	2.0	4 (2)	0	0	0	2 (2)	0
A7	0.5	0	0	0	0	5 (3)	2 (2)
A9	2.0	2 (2)	0	3 (3)	0	0	0

* See footnote to Table 1.

A number of investigators (1-7, 9, 10, 13, 14, 18) have reported that anionic polymers counteract the inhibitory action of agar polysaccharides. However, they used Noble agar, which we have previously shown to cause an adverse reaction

with monkey kidney cells, as well to have an inhibitory action on certain viruses (18). Nagai and Hammon recently (8) confirmed these findings on the deleterious effects of Noble agar. If Difco agar is used, the problems associated with

plaque-inhibitory substances in agar are decreased considerably. In this study, we found that the inhibitory effect of Difco agar when it existed for enterovirus, could usually be relieved to a greater degree by $MgCl_2$, cysteine, or pancreatin, than by anionic polymers.

Of the 41 enteroviruses tested, $MgCl_2$ enhanced plaque development of 24 strains; cysteine, 20 strains; pancreatin, 8 strains; DEAE dextran, 4 strains; dextran sulfate, 7 strains; and protamine sulfate, 10 strains.

Zajac and Crowell (19) reported that pancreatin rendered HeLa cells resistant to infection with group B coxsackieviruses. In our present study, the presence of pancreatin in the overlay medium had no inhibitory effects on these viruses. However, we used monkey kidney cells exclusively, and the receptors of monkey kidney cells may be more resistant to pancreatin than those of HeLa cells.

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