

Use of DEAE Dextran in Agar Overlays To Enhance Size of ECHO Virus Plaques

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THE PLAQUE inhibition test is an important technique in studying the antigenic interrelationships of ECHO viruses. In this test, the virus must produce discrete, countable plaques. When the standard agar overlay technique is used, many of the ECHO viruses produce minute, faint plaques on monolayers of rhesus monkey kidney (1, 2). Takemoto (3) has shown that agar releases a sulfated polysaccharide, which inhibits the growth of wild-type encephalomyocarditis virus, and that this inhibitor causes the reduction of the plaque size of some viruses. Takemoto (4) also demonstrated that this inhibition could be overcome by the addition of diethylaminoethyl dextran (DEAE-D). Therefore we decided to add DEAE-D to the agar overlay medium to see if DEAE-D facilitated the development of larger ECHO virus plaques with ECHO virus types 1 through 27.

Materials and Methods

Viruses. All ECHO viruses used were prototype strains (5) which had been purified by three terminal plaque passages. High-titer virus stocks—in the range of 10^6 TCD₅₀ to 10^8

TCD₅₀ per milliliter—were obtained by growing the virus in cultures of rhesus monkey kidney.

Plaquing technique. The plaquing technique was essentially that of Dulbecco and Vogt (6). Five milliliters of a suspension of trypsinized rhesus monkey kidney cells was placed in disposable plastic 60 mm. petri dishes. The cell suspension contained 300,000 cells per milliliter, suspended in Earle's saline with 0.5 percent lactalbumin hydrolysate and 5 percent calf serum. Petri dishes were incubated 6 to 7 days at 37° C. in an atmosphere of 3–5 percent CO₂; at the end of this period there was usually a complete cell sheet.

The nutrient agar overlay consisted of three parts, which are mixed to make up the final overlay. The first contained 2.2 percent Difco purified agar in distilled water; the second contained 0.075 percent neutral red solution in distilled water, sterilized by filtration (A); and the third, or 2X nutrient medium, contained 1 percent lactalbumin hydrolysate, 0.2 percent yeast extract, and 0.2 percent bovine albumin fraction in 2X Earle's solution with 0.44 percent NaHCO₃. The 2X nutrient overlay was saturated with CO₂ to prevent precipitation and was then sterilized by positive filtration. Then, 400,000 units of penicillin, 200,000 units of streptomycin, and 5.0 mg. of amphotericin B were added per liter.

In preparing the final agar overlay medium, the agar was melted, then cooled and held at

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45° C. One part of 0.075 percent neutral red solution was combined with nine parts of warm agar. Equal parts of 45° C. neutral red agar and 45° C. 2X nutrient medium were combined; 5 ml. of this mixture was used to overlay. If the overlay was to contain DEAE-D, the DEAE-D was added to the 45° C. neutral red agar mixture. The final overlay contained 50 mg. of DEAE-D per milliliter.

Testing for comparative plaque size. Ten-fold dilutions were made, and six plates were inoculated at each dilution with 0.2 ml. of virus per plate. After 30 minutes at room temperature three plates were overlaid with nutrient agar containing 50 µg. of DEAE-D per milliliter; three plates were overlaid with nutrient agar without DEAE-D. All inoculated plates were incubated at 37° C. in an atmosphere of 3-5 percent CO₂. Seven days after inoculation, the plates were examined, and the size, shape, and degree of clearness of the plaques were recorded. In overlays both with and without

DEAE-D, the batch of monkey kidney cells greatly influenced the size of the plaques. Therefore, all the ECHO virus types were tested on the same day with the same batch of monkey kidney monolayers. Each monolayer was a complete cell sheet on which the cells were apparently healthy and free of simian agents. Absence of simian agents was of great importance, as DEAE-D not only enhanced the formation of some ECHO virus plaques, but also greatly increased the size of simian virus plaques. In fact, about half of all the tests of antigenic studies had to be discarded because of the presence of simian plaques in the uninoculated controls.

Results

The results shown in the table are the mean of two 7-day readings on monolayers containing no obvious adventitious virus. The ECHO virus types in which the plaques were visibly enhanced by the presence of DEAE-D are in

Effects on plaque morphology of ECHO viruses of incorporating DEAE-D in the agar overlay

ECHO virus type	Plaque morphology 7 days after inoculation of monkey kidney monolayers					
	DEAE-D in overlay			No DEAE-D in overlay		
	Size (mm.)	Clarity	Regularity	Size (mm.)	Clarity	Regularity
1	6-7	Clear	Irregular	3-4	Clear	Irregular.
2	6-7	do	do	1-2	Faint	do.
3	7-8	do	do	1-2	do	do.
4P	2-4	Faint	do	2-3	do	do.
5	3-4	Clear	do	1-2	do	do.
6	7-8	do	do	1-2	Clear	do.
7	9-12	do	Round	9-12	do	Round.
8	5-6	do	Irregular	3-4	do	Irregular.
9	3-5	Faint	do	1	do	do.
11	2-3	do	do	2-3	do	do.
12	12-18	Clear	Round	10-15	Clear	Round.
13	2-4	Faint	Irregular	1	Faint	Irregular.
14	2-3	do	do	2-3	do	do.
15	3-5	do	do	2-3	do	do.
16	1-2	do	do	1-2	do	do.
17	2-3	do	do	1-2	do	do.
18	2-3	do	do	2-3	do	do.
19	9-12	Clear	Round	8-12	Clear	Round.
20	1-2	Faint	Irregular	<1	Faint	Irregular.
21	1-2	do	do	1-2	do	do.
22	2-3	do	do	2-3	do	do.
23	2-3	do	do	2-3	do	do.
24	3-4	do	do	2-3	do	do.
25	2-3	do	do	1	do	do.
26	2-3	do	do	<1	do	do.
27	1-2	do	do	1-2	do	do.

NOTE: Boldface type indicates substantial enhancement by the presence of DEAE-D.

boldface type. For 38 percent of the ECHO viruses, the plaques increased in size in plates overlaid with agar containing DEAE-D as compared with plates not receiving DEAE-D. As can be seen from the table, the small plaques were usually the ones that were enhanced. The presence of DEAE-D in the agar overlay enlarged the plaques of ECHO viruses types 1, 2, 3, 5, 6, 9, 13, 20, 25, and 26; types 2, 3, 6, and 9 showed the greatest enhancement in plaque size. The plaques produced by types 2, 3, and 5 changed from faint to clear in the presence of DEAE-D. Of special interest is the enhancement of the plaque size of types 9, 13, 20, 25, and 26. Plaques of these types originally had been so small—1 mm. or less—that plaque work with them was extremely difficult.

Enhancement of plaque size is most important and, fortunately, all types of ECHO virus will produce discernible plaques in the presence of DEAE-D. The addition of DEAE-D did not increase the total number of plaques for any of the ECHO viruses. The presence of DEAE-D in the agar did not enhance or suppress the inactivating value of the antisera and apparently did not damage the layer of monkey kidney cells. In fact, the only obvious effects of its presence were changes in the plaque characteristics of some of the ECHO virus types.

Discussion

The addition of DEAE-D to the agar overlay and the concurrent increase in plaque size made it possible to proceed with antigenic studies using plaque reduction. The enhanced plaque characteristics may be advantageous in many instances, such as in separation of mixed viruses, terminal plaque purification of viruses, quantitative evaluation of serums by plaque reduction, isolation of special genetic types, differentiation of strains within a type by plaque technique, as described by Wecker (7) or McBride (8), and in any other area in which plaque procedures are used.

As previously mentioned, the addition of DEAE-D to agar apparently augments the size of the plaques produced by simian virus. During a 6-month period, 49 different batches of monkey kidney cells were used. In 25 (51 percent) of these batches, plaques were discernible

in the uninoculated control plates overlaid with DEAE-D. Plaque formation was seen in only 11 (22 percent) of the the uninoculated control plates overlaid without DEAE-D. Plaque formation was a vexing occurrence in many of the plates during our studies, but it actually might be useful in other circumstances. Simian agents are a common contaminant of cultures grown in monkey kidney tissue. In vaccines produced by growing the virus in such cultures, these agents create difficulties. Addition of DEAE-D to plated material would facilitate visualization of these adventitious agents and could be useful for plaque purification. In general, use of DEAE-D could simplify studies of these simian agents.

Summary

Many ECHO virus strains produce minute, faint plaques on monolayers of rhesus monkey kidney when the plaque inhibition test is performed by standard overlay technique. Plaques of some types are so small or so faint that work with them is difficult.

We tested the effect on plaque size of overlaying plates of ECHO virus types 1 through 27 with agar containing diethylaminoethyl dextran (DEAE-D). The presence of DEAE-D in the agar overlay enlarged the plaques of ECHO viruses types 1, 2, 3, 5, 6, 9, 13, 20, 25, and 26. Addition of DEAE-D also enhanced the size of contaminating simian viruses.

All types of ECHO viruses produced discernible plaques in the presence of DEAE-D, and its presence in the agar apparently did not harm the monkey kidney cell layer. Its only obvious effect was to change the plaque characteristics of some ECHO virus types.

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of poliovirus strains within the same type. *Virology* 10:376 (1960).

- (8) McBride, W. D.: Antigenic analysis of polio-
viruses by kinetic studies of serum neutraliza-
tion. *Virology* 7:45 (1959).

EQUIPMENT REFERENCE

- (4) Millipore membrane filter, Millipore Filter Corp.,
Bedford, Mass.

Legal note . . . Fluoridation

Fluoridation of public water supply by action of the city council held valid exercise of city's police power. *Hall v. Bates, et al.*, 148 S.E. 2d 345 (S.C. 1966)

The plaintiff, Hall, sought to enjoin the city of Columbia, S.C., from fluoridating its water supply on the grounds that such action violated the due process and equal protection clauses of both the Federal and his State's constitutions. The city council, after holding a public hearing, had concluded that its water supply should be fluoridated.

The lower court found that placing fluoride in the city's water supply was beneficial, rather than harmful, to the health of its citizens and that fluorides would not be harmful to the plaintiff notwithstanding his arthritic condition.

The Supreme Court of South Carolina, noting it had never passed on this issue, affirmed the lower court's decision, holding that the action taken by the city was a constitutional exercise of the police power vested in it in that such action was reasonably necessary to the public health. The court concluded that the means used and the extent of any interference with rights of the plaintiff were reasonably necessary to the accomplishment of the purpose sought to be attained.

In the course of its opinion, the supreme court expressly rejected the plaintiff's argument that the action taken was an improper exercise of the police power because (a) tooth decay is not a communicable disease, (b) only children are benefited, and (c) it subjects all users to mass medication without choice by concurring with "those well-reasoned" precedents which ". . . (1) sustain the right of municipalities to adopt reasonable measures to improve or protect the public health, even though communicable or epidemic diseases are not involved; (2) hold that the benefits of fluoridation which carry over into adulthood absolve such programs of the charge of being class legislation; and, (3) conclude that fluoridation programs, even if considered to be medication in the true sense of the word, are so necessarily and reasonably related to the common good that the rights of the individual must give way."—HOWARD WALDERMAN, attorney, *Public Health Division, Office of the General Counsel, Department of Health, Education, and Welfare.*