GROWTH, PLAQUE PRODUCTION, AND CATIONIC STABILIZATION OF RHINOVIRUS TYPE 1 (ECHOVIRUS 28)

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ABSTRACT

BEHBEHANI, ABBAS M. (Baylor University College of Medicine, Houston, Tex.), AND L. H. LEE. Growth, plaque production, and cationic stabilization of rhinovirus type 1 (echovirus 28). *J. Bacteriol.* **88**:1608-1611. 1964.—A variety of conditions for the growth of echovirus 28 were investigated. The virus grew more readily and to the highest titer when secondary MK cells maintained in low bicarbonate and rolled at 33 C were employed. Viral plaques were produced in human diploid (WI-38) cell cultures. The virus was protected from thermal inactivation (50 C for 1 hr) by 1 M MgCl₂. For storage of the virus at refrigerator and room temperature, the use of 1 M MgCl₂ is also of advantage.

Echovirus 28 has properties indicating that it should be included among the rhinoviruses, a subgroup of the picornaviruses (International Enterovirus Study Group, 1963). Strains of this virus were first isolated in 1956 from respiratory infections of both adults and children (Price, 1956; Pelon et al., 1957). Subsequent studies have shown that this virus is one of the etiological agents of the common cold, and that it can produce a common cold-like illness in volunteers (Hamre and Procknow, 1961; Jackson, Dowling, and Muldoon, 1962; Taylor-Robinson and Tyrrell, 1962; Jackson, Dowling, and Mogabgab, 1960).

It has been reported that the multiplication and the cytopathic effects of this virus could be greatly enhanced by the use of secondary monkey kidney cell cultures maintained in media containing low bicarbonate concentration and rolled at 33 C (Pelon and Mogabgab, 1959; Holper et al., 1960; Mogabgab and Holmes, 1961; Pelon, 1961; Sharma and Werner, 1962). However, the

relative role of each of the above-mentioned factors, in the growth of echovirus 28, has not been systematically investigated, and this Centre has had reports of difficulty in propagating the virus from a number of laboratories. Furthermore, no data on the cationic stabilization of this virus are available, and only negative reports on the plaque production have been published (Pelon, 1961; Pelon and Mogabgab, 1958). The present investigation was undertaken to study these and other related problems.

MATERIALS AND METHODS

Monkey kidney (MK) cells. Kidney cells from immature grivet or rhesus monkeys were grown in Melnick's medium A, as described in detail elsewhere (Melnick, Wenner, and Rosen, 1964). Secondary cell cultures were prepared by trypsinizing primary cultures after 7 days of growth in 16-oz prescription bottles. From each 16-oz bottle of primary kidney culture, about 14 plaque bottles (1 oz) or 35 culture tubes of secondary cells were inoculated. These secondary cultures were ready for inoculation within 3 to 6 days.

Human diploid (WI-38) cells. Tissue-culture tubes and plaque bottles of this cell line at passages ranging from the 28th to 32nd were prepared, according to the method described by Hayflick and Moorhead (1961).

Maintenance medium. The maintenance medium for both primary and secondary MK cells was Melnick's medium B containing 0.06% sodium bicarbonate with a final pH value of 7.1 to 7.2. Inoculated cultures were rolled at 33 C. For comparison purposes, however, some inoculated cultures were maintained in medium containing 0.22% sodium bicarbonate. These cultures, in turn, were either rolled or kept stationary at 37 C or at 33 C. No protein was added unless the cultures were held longer than 5 days, when 0.1% skim milk or 1.0% calf serum was added. The maintenance medium for

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the human diploid cell line was Eagle's basal medium containing 2% calf serum with a final pH value of 7.2.

Plaque overlay. The "high magnesium" agar overlay described by Wallis, Melnick, and Bianchi (1962), but containing 0.07% sodium bicarbonate, 2% calf serum, and 1% agar, was used in these experiments. Fully grown cell monolayers in 1-oz bottles were inoculated with 0.2 ml of stock virus diluted 10^{-1} to 10^{-5} and adsorbed at 33 C for 1 to 2 hr. The bottles were then overlaid with a thin layer (about 4.0 ml per bottle) of the above medium without neutral red and incubated at 33 C for 4 days. After this incubation period, another thin layer of the overlay medium, but this time containing 0.0017% neutral red, was added. The bottles were immediately covered with aluminum foil and reincubated at 33 C.

Virus. A stock of echovirus 28, strain 2060, grown in secondary grivet monkey kidney cells rolled at 33 C, was used for the experiments reported here. The titer of this virus was $10^{5.7}$ TCID₅₀ per ml. For the cationic stabilization experiments, 0.75 ml of virus was added to 0.25 ml of 4 M MgCl₂, to obtain virus in 1 M MgCl₂. Control virus for these experiments was similarly diluted with distilled water.

RESULTS

Comparative studies on the growth of virus. The effects of variations in temperature (37 or 33 C), bicarbonate concentration (0.22% or 0.06%), and incubation position (stationary or rolled) on the growth of this virus in both primary and secondary grivet monkey kidney cells grown in 1-oz bottles (10^6 cells) were investigated. The inoculum for all cultures was 0.2 ml of 10^{-1} dilution of the stock virus. Before inoculation, the medium was thoroughly removed. The virus was added and adsorbed in a stationary position at either 37 C or 33 C (according to the temperature at which the virus was to be grown) for 1 hr, and then the appropriate maintenance medium was added. The cultures were incubated in a stationary position or rolled at both 37 and 33 C. They were examined twice daily for the appearance of cytopathic effects (CPE). Cultures showing 3+ (75%) CPE were frozen and thawed once, pooled, and centrifuged at 3,000 rev/min for 30 min. The supernatant fluid was separated and kept frozen at -20 C until titrated. All

titrations were done within 1 week after harvesting the virus in secondary MK tubes maintained in low bicarbonate concentration (0.06%), and rolled at 33 C. The original cultures were observed for 9 days, at which time they were harvested. Nonspecific cellular degeneration may occur in secondary MK cells at this time, especially in cultures rolled at 37 C. The results of these experiments are summarized in Table 1.

It can be seen that the optimal growth of the virus occurred in cultures maintained in low bicarbonate concentration and rolled at 33 C. Under these conditions, primary MK cultures were as susceptible as secondary cultures. The results also indicate that variations in temperature, incubation position, and bicarbonate concentration affect the growth of virus more significantly in primary cultures than in secondary cultures. Furthermore, in both primary and secondary cultures, temperature appeared to be the most important factor, followed (in order of decreasing importance) by incubation position and bicarbonate concentration. In almost every instance, the growth of the virus at 33 C was faster, and the titers of the harvests obtained were higher, than the corresponding virus grown at 37 C. Rolled cultures produced virus harvests of higher titers than stationary cultures when both were incubated at 33 C. On the other hand, at 37 C, the stationary cultures yielded harvests of higher titers than rolled cultures. Finally, a low bicarbonate concentration in the medium permitted, in every case, better virus growth than high bicarbonate concentration.

Plaque production. Several attempts at plaquing this virus in primary and secondary MK (both rhesus and grivet) cultures under a variety of experimental conditions were unsuccessful. Since this virus grows readily and produces marked CPE in human diploid cultures, its capability to produce plaques in this cell line was investigated. Bottles (1 oz) showing confluent cell sheets were inoculated with the virus, adsorbed, and overlaid, according to the procedure described above. Plaques became visible 48 to 72 hr after the second agar overlay and were counted 3 to 4 days after adding the second overlay. Plaques were hazy, irregular, and about 1 to 2 mm in diameter. Addition of either sodium dextran sulfate (50 μ g/ml) or diethylaminoethyl-dextran (100 μ g/ml) to the first agar overlay, as recommended by Kisch, Webb, and Johnson

TABLE 1. *Degree of cytopathic effect (CPE)* of echovirus 28 in monkey-kidney cultures and titers of harvests*

MK cultures	Incubation position	Temp	Per cent NaHCO ₃	Days after inoculation								Titer of harvest† (log TC ₅₀ per ml)
				2	3	4	5	6	7	8	9	
Primary	Rolled	C	0.06			3+						5.5
			0.22				3+					5.2
		37	0.06								3+	3.2
			0.22								±	<1
	Stationary	33	0.06			3+						4.5
			0.22								1+	2.7
		37	0.06								2+	3.2
			0.22								1+	1.5
Secondary	Rolled	33	0.06		3+							5.7
			0.22				3+					5.2
		37	0.06				3+					4.2
			0.22								1+	2.5
	Stationary	33	0.06			3+						5.2
			0.22						3+			3.7
		37	0.06				3+					4.7
			0.22								2+	3.7

* CPE is expressed on the following scale: 1+ = 25% of the cells show degeneration; 2+ = 50% show degeneration; 3+ = 75% show degeneration; ± = questionable cell damage.

† Virus was harvested on day of maximal CPE as indicated in table.

TABLE 2. *Stabilizing effect of 1 M MgCl₂ on echovirus 28**

Expt	Temp of storage	Time	Log TC ₅₀ per ml*	
			Virus in 1 M MgCl ₂	Control virus
1	C			
	-20	10 days	5.5	5.7
	+4	10 days	4.2	3.7
2	25 to 30	10 days	3.5	2.7
	-20	10 days	4.7	4.5
	+4	10 days	4.2	3.5
	25 to 30	10 days	3.2	2.7
	50	60 min	4.5	3.2

* Two different lots of virus having different titers were used in the two experiments. The virus used for the heating experiment was the same as the one used in experiment 2.

(1964) for some rhinoviruses, did not cause any change in the size or number of plaques. Echovirus 28, grown in either human diploid or secondary MK cultures, was titrated by this

plaque assay method in four different experiments, and titers of plaque-forming units corresponding to those of the tube method were obtained.

Cationic stabilization. That echovirus 28 is not completely inactivated by heating at 50 C for 30 min was shown by Ketler, Hamparian, and Hilleman (1962). We obtained similar results (a reduction of 1.5 to 2.0 log in titer) in the heating experiments with our stock virus. However, when virus was suspended in 1 M MgCl₂, no appreciable loss of titer occurred after heating at 50 C for 1 hr.

To test the stabilizing effect of 1 M MgCl₂ on this virus during storage at various temperatures, the following experiment was undertaken. Samples of stock virus in 1 M MgCl₂ and control virus without MgCl₂ were stored at room temperature (25 to 30 C), 4 C, and -20 C for 10 days and then titrated simultaneously in secondary MK cells under optimal conditions (low bicarbonate and rolling at 33 C). Upon addition of MgCl₂, the pH of the virus suspension usually dropped from 7.1-7.2 to 6.4-6.5. At the end of the storage period, however, the pH was 6.6 ±

0.1. The results are presented in Table 2. It can be seen that, although 1 M MgCl_2 did not bring about complete stabilization of the virus at 4 C and room temperature, better preservation at these two temperatures could be achieved with 1 M MgCl_2 .

DISCUSSION

In recent years, much interest has been focused on echovirus 28 as one of the etiological agents of the common cold. The virus has been included in upper respiratory disease vaccines currently under investigation in the United States and abroad (Mufson et al., 1963). It seems that an incubation temperature of 33 C, rolling, and maintaining the infected cultures under low bicarbonate (pH 7.0 to 7.2) produce virus of high titer, in confirmation of the findings of others (Pelton and Mogabgab, 1959; Holper et al., 1960; Mogabgab and Holmes, 1961; Pelton, 1961).

Experiments with MgCl_2 have shown that this virus, when suspended in 1 M MgCl_2 , is stabilized after heating at 50 C for 1 hr. In this property, echovirus 28 is similar to members of the enterovirus group (Wallis and Melnick, 1962). However, this virus differs from enteroviruses in that, when heated without MgCl_2 at 50 C for 1 hr, only 1 to 2 log reduction in titer occurred, as compared to 4 to 5 log with other enteroviruses. For preservation of this virus during temporary storage and long-distance shipment at icebox or room temperature, suspending the virus in 1 M MgCl_2 is of definite advantage.

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