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Susceptibility of the LLC-MK_2 line of monkey kidney cells to human enteroviruses

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The isolation and identification of human enteroviruses in tissue culture is often a laborious and expensive procedure. In many laboratories the tests are made in primary or secondary monkey kidney cultures despite the disadvantages associated with the use of these tissues. Adequate supplies may be difficult to obtain when required and the maintenance of a monkey colony is expensive. A further disadvantage of primary monkey kidney cultures is the not infrequent presence of adventitious agents (Tobin, 1960). The use of a continuous line of cells eliminates these difficulties. If it is to be a satisfactory substitute, a continuous cell line should be at least as susceptible to the virus under investigation as primary cultures.

This report contains the results of experiments planned to determine whether the continuous line of LLC-MK_2 cells (Hull, Cherry & Johnson, 1956; Hull, Cherry & Tritch, 1962) would be a suitable replacement for primary rhesus monkey kidney cultures for the isolation and identification of enteroviruses. The relative susceptibility of LLC-MK_2 and primary monkey kidney cells to prototype enteroviruses before and after passage in LLC-MK_2 is shown. This is followed by the number of isolations recorded when faecal extracts were inoculated into both types of tissue. Finally, the two tissues are compared when used for the identification of enteroviruses by neutralization with specific antisera.

MATERIALS AND METHODS

Virus strains

The prototype ECHO, Coxsackie B and poliovirus strains used are listed in Table 1. With the exception of ECHO 21, which was grown in human amnion cultures, all the strains were propagated in primary rhesus monkey kidney tissue. When extensive cytopathic effect (CPE) was present the cultures were frozen and thawed, and centrifuged at about 100 *g* to remove the cell debris. The supernatant was stored at $-30°$ C. until required. These supernatant fluids were passaged twice in LLC-MK_2 cells before titrations for comparative infectivity were made. Faecal extracts from which cytopathic agents had previously been isolated were removed from storage and re-examined in LLC-MK_2 and primary

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monkey kidney tissues. Cultures, in which no CPE was present after 10 days, were regarded as negative only after further passage had failed to reveal a cytopathic agent.

Tissue cultures

Primary monkey kidney. Kidneys from freshly killed rhesus monkeys were trypsinized by a method based on that described by Rappaport (1956). Cultures were made in tubes ($4 \times \frac{1}{2}$ in.) incubated at 36–37° C. in stationary racks. Growth medium consisted of Hanks's balanced salt solution containing 0·5% lactalbumin hydrolysate, 2% bovine serum, 0·05% sodium bicarbonate and antibiotics. Maintenance fluid was medium 199 containing 0·22% sodium bicarbonate and antibiotics.

LLC-MK$_2$ cells. The continuous line of LLC-MK$_2$ cells in the 285th passage was received from Dr R. N. Hull, Lilly Research Laboratories, Indianapolis. All the cultures used in this investigation were made from cells between the 298th and 328th passage. Stock cultures were grown in flat bottles on medium 199 containing 1% horse serum, 0·15% sodium bicarbonate and antibiotics. Subcultures were made when the cell sheet was confluent. The cells were removed from the glass by a 0·02% solution of Sequestric acid (EDTA) and incubation at 37° C. for 15–30 min. followed by agitation. The cell suspension was centrifuged slowly and the deposit resuspended in growth medium. Each tube received 1 ml. of cell suspension containing approximately 10^5 cells. Incubation was at 36–37° C. in stationary racks. Before the cultures were inoculated, the growth medium was replaced by medium 199 containing 0·22% sodium bicarbonate and antibiotics. Cultures were used within 4 days of preparation.

Virus titrations

Serial 0·5 log dilutions of virus suspension were prepared in maintenance medium. At each dilution 0·1 ml. volumes were transferred to four culture tubes containing 1 ml. of maintenance medium. Cultures were incubated at 36–37°C. in stationary racks and examined at intervals for CPE. Final readings were recorded after 7 days when any culture showing distinct CPE was considered to be positive for infective virus. The method of Reed & Muench (1938) was used to estimate 50% infectivity end-points which are recorded as the log tissue culture infective dose per 0·1 ml. (log TCD 50/0·1 ml.). Logarithms are expressed to the base 10.

Virus identification

Viruses were identified in neutralization tests with specific antisera. The method employed was that described by Hambling, Davis & Macrae (1963) in which composite antiserum pools were used. Agents were typed in both LLC-MK$_2$ and primary rhesus monkey kidney cultures whenever the tissues supported sufficient virus growth to produce at least 1000 TCD 50/0·1 ml. If either tissue yielded insufficient virus, at least three passages were made before the tissue was rejected as unsuitable for use in identification of the agent.

RESULTS

Virus suspensions which had been propagated in primary rhesus monkey kidney tissue (pMK) were passaged twice in LLC-MK$_2$ cultures. Infectivity titrations of this second passage material, and also of the virus suspension before passage in LLC-MK$_2$ cells were made in both pMK and LLC-MK$_2$ cultures. Distinct cytopathic effect in LLC-MK$_2$ cultures was produced by thirty-four of the thirty-

Table 1. *Comparison of infectivity titrations of enteroviruses in primary rhesus monkey kidney (pMK) and LLC-MK$_2$ cultures before and after two passages in LLC-MK$_2$ cells*

		Titration of virus grown in pMK†		Titration of virus after two passages in LLC-MK$_2$†	
Virus*	Strain	In pMK	In LLC-MK$_2$	In pMK	In LLC-MK$_2$
ECHO 1	Farouk	6·8	6·2	6·9	6·2
ECHO 2	Cornelis	6·2	6·0	6·3	5·8
ECHO 3	Morrisey	5·6	5·8	5·4	5·9
ECHO 4	Pesascek	4·4	2·8	4·8	3·3
ECHO 5	Noyce	6·5	6·1	7·2	7·0
ECHO 6	D'Amori	6·8	6·1	6·9	6·3
ECHO 7	Wallace	5·8	5·8	6·8	6·9
ECHO 9	Hill	6·9	6·0	7·1	6·9
ECHO 11	Gregory	6·8	5·8	7·0	6·5
ECHO 12	Travis	6·7	6·1	6·3	7·0
ECHO 13	11-4D	4·2	2·9	5·3	4·8
ECHO 14	Tow	4·5	3·3	5·3	5·3
ECHO 15	Charleston	5·0	4·3	4·6	4·8
ECHO 17	CHHE-29	5·3	3·3	6·1	3·8
ECHO 19	Burke	7·3	7·3	7·0	6·5
ECHO 20	JV-1	5·5	4·9	6·1	5·9
ECHO 22	Harris	5·2	1·9	5·1	2·3
ECHO 24	DeCamp	4·7	3·6	6·3	5·2
ECHO 25	JV-4	5·7	3·8	6·2	4·6
ECHO 26	Coronel	5·3	4·7	6·8	6·1
ECHO 27	Bacon	5·7	3·0	7·1	4·3
ECHO 29	JV-10	7·0	5·3	7·5	5·7
ECHO 30	Bastianni	6·1	5·0	6·0	5·3
ECHO 31	Caldwell	5·4	1·8	5·2	2·2
ECHO 32	PR 10	5·3	4·5	6·8	5·1
Coxsackie B1	P.O.	5·8	6·2	6·2	6·4
Coxsackie B2	Ohio (Red)	5·0	4·4	7·0	7·0
Coxsackie B3	Nancy	6·2	6·3	6·9	7·0
Coxsackie B4	J.V.B.	5·5	5·8	6·5	6·3
Coxsackie B5	Faulkner	5·8	5·8	7·0	6·5
Coxsackie B6	Schmitt	5·3	6·0	6·8	7·2
Poliovirus type 1	Mahoney	6·1	5·8	7·0	6·8
Poliovirus type 2	YSK	5·8	6·2	6·2	7·0
Poliovirus type 3	Saukett	6·3	5·4	7·2	5·8

* ECHO types 16 (Harrington), 18 (Metcalf), 21 (Farina) and 23 (Williamson) did not cause distinct CPE in LLC-MK$_2$ cells.

† Expressed as log TCD50/0·1 ml.

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eight enteroviruses examined; only ECHO types 16, 18, 21 and 23 failed to show this effect.

The Coxsackie B and polioviruses readily caused CPE in LLC-MK_2 cells and with the possible exception of poliovirus type 3, showed no great difference in titre when titrated in both tissues (Table 1). The poliovirus type 3 (Saukett) both before and after passage in LLC-MK_2 cells, showed higher titres in pMK than in LLC-MK_2 cultures. Further investigations are necessary to determine whether this is a real difference and due to the LLC-MK_2 cells being less susceptible than the pMK to this virus strain.

Table 2. *Isolation and identification of enteroviruses from faecal extracts in primary rhesus monkey kidney (pMK) and LLC-MK_2 cultures*

Isolates		No. isolated and identified in		
Virus and type represented	No.	pMK and LLC-MK_2	pMK only	LLC-MK_2 only
Poliovirus (types 1, 2 or 3)	11	11	—	—
Coxsackie B (types 2, 3, 4 or 5)	15	15	—	—
ECHO (types 1, 3, 6, 7, 9, 11, 13, 14, 16, 17 or 21)	57	23	33	1
Totals	83	49	33	1

Variation in infectivity titres was obtained when the ECHO viruses, propagated in pMK, were titrated in the two tissues (Table 1). Seven viruses (ECHO types 4, 17, 22, 25, 27, 29 and 31) had infective virus concentrations of at least 1·5 log greater in pMK than in LLC-MK_2 cells and this difference persisted after the agents had been passaged twice in LLC-MK_2 cultures.

Enteroviruses were isolated from eighty-three faecal extracts (Table 2). This number comprised fifty-seven members of the ECHO group (types 1, 3, 6, 7, 9, 11, 13, 14, 16, 17 or 21), fifteen Coxsackie B viruses (types 2, 3, 4 or 5) and eleven polioviruses (types 1, 2, or 3). All Coxsackie B and polioviruses were isolated in both LLC-MK_2 and pMK cultures. In contrast, only twenty-three of the fifty-seven ECHO viruses were isolated in both tissues and thirty-three caused CPE only in pMK. All the agents were typed by neutralization tests in the tissue in which they were isolated. Those which caused CPE in the two tissues were identified in both; in no instance did the results differ.

The time for distinct CPE to appear in the tissues inoculated with faecal extracts varied. In general, the ECHO viruses took longer to produce CPE in LLC-MK_2 cells than in pMK, whereas the Coxsackie B and polioviruses usually caused CPE in both tissues at about the same time.

DISCUSSION

In 1955 the continuous line of LLC-MK_2 cells was derived from a pool of cells prepared by trypsinization of kidneys from six rhesus monkeys (Hull *et al.* 1962). It has advantages over primary monkey kidney cultures in being free from

adventitious viruses, readily available, and economical in serum requirements as it is grown on a medium containing only 1% horse serum. If it could be shown to be as susceptible to viruses as primary cell cultures it should provide an excellent replacement for them.

In this investigation thirty-eight prototype strains of human enteroviruses and eighty-three agents isolated from faecal extracts have been examined in LLC-MK$_2$ and primary rhesus monkey kidney cultures. The results indicate that for some laboratory investigations involving Coxsackie B and polioviruses LLC-MK$_2$ cells could be an adequate substitute for the primary cultures. For example, it may prove useful as the tissue culture system in tests for the estimation of neutralizing antibodies to the Coxsackie B viruses.

The prototype strains of ECHO types 16, 18, 21 and 23 failed to produce CPE in LLC-MK$_2$ cells. If other strains of these types behave similarly, this lack of CPE may serve as a preliminary indication that an unknown agent belongs to one of these four types. The LLC-MK$_2$ cell line was susceptible to the remaining prototype ECHO viruses, but suspensions of types 4, 17, 22 and 31, despite two passages, only contained low concentrations of virus when titrated in LLC-MK$_2$ cultures.

It is possible that the susceptibility of a continuous line of cells may change after subculture. In this study extensive experiments to investigate this were not made. However, the suspensions of three viruses which had been titrated in 298th or 299th passage cells were stored at $-30°$ C. and re-titrated at a later date in 321st passage cells. No significant differences in the titres were observed.

The faecal extracts examined were from specimens sent to the laboratory for virus isolation so they contained a high percentage of the agents which were prevalent at that time. This was especially noticeable in the ECHO group where there was a predominance of ECHO type 6 and to a lesser extent ECHO type 9 viruses. As many of the ECHO type 6 viruses caused CPE in pMK but not in LLC-MK$_2$ cultures, the isolation rate of ECHO viruses in LLC-MK$_2$, when ECHO type 6 is not prevalent, may well be higher than that shown in Table 2. However, as the extracts tested were from the actual specimens which the laboratory had been requested to examine, it seemed justifiable to use this material to evaluate the use of LLC-MK$_2$ cells for virus isolations.

Prime strains of ECHO type 6 virus are recognized (Melnick, 1957) and variation in the susceptibility of cultures to ECHO type 6 viruses has been reported. Pal, McQuillin & Gardner (1963) described the isolation of eighty-nine ECHO type 6 viruses in Hep 2 cells of which only thirty-seven were also isolated in primary cynomolgos monkey kidney cultures. Hsiung (1962) found that the prototype (D'Amori) ECHO type 6 virus failed to cause CPE in Hep 2 cells. During the time faecal specimens were collected, prime strains of ECHO type 6 virus were present in the population so it is possible that the ECHO type 6 viruses which were isolated in pMK but not in LLC-MK$_2$ cells fall into this category, whereas the few which cause CPE in both tissues resembled the prototype strain. This would be supported by the susceptibility of LLC-MK$_2$ cells to the prototype strain as shown in Table 1.

The identification of forty-nine enteroviruses by neutralization tests in both

pMK and LLC-MK_2 cultures showed complete agreement, so it appears that the continuous line would be of value for this purpose. It is satisfactory for the isolation of Coxsackie B and polioviruses. The variation in susceptibility to the ECHO viruses makes it advisable to use the LLC-MK_2 line in conjunction with other culture systems for maximum isolation rates.

SUMMARY

The relative susceptibility of the LLC-MK_2 cell line and primary rhesus monkey kidney cultures to thirty-eight prototype strains of human enteroviruses is described; of these strains only ECHO types 16, 18, 21 and 23 failed to cause CPE in the continuous cell line. The efficiency of the two tissues for the isolation of enteroviruses from faecal extracts is compared. The results show that the LLC-MK_2 cell line is very satisfactory for the isolation of Coxsackie B and polioviruses, but not so useful for the isolation of ECHO viruses. The identification of enteroviruses by neutralization tests in LLC-MK_2 cells is successful.

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