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Susceptibility of the VERO line of African green monkey kidney cells to human enteroviruses

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SUMMARY

The relative susceptibility of VERO cells and primary rhesus monkey kidney cells to 47 prototype strains of human enteroviruses is described. Of these strains, types 4, 14, 16, 17, 18, 21, 31 and 34 and Coxsackie virus A 9 failed to cause CPE in the VERO cells whilst only one, echovirus type 34, failed to cause CPE in the monkey kidney cells.

A comparison is given of the efficiency of the two cell cultures for enterovirus isolation from clinical material. Results show that VERO cells are as useful as primary monkey kidney for the isolation of Coxsackie B viruses but less satisfactory for isolating echoviruses. They are satisfactory for the isolation of single types of poliovirus and appear to be more satisfactory than primary monkey kidney cells for the isolation of mixtures of polioviruses. The identification of enteroviruses by neutralization tests in VERO cells is successful.

INTRODUCTION

The isolation and identification of human enteroviruses is done by many laboratories in primary or secondary rhesus monkey kidney cultures. There are however, certain disadvantages associated with these tissues such as their varying sensitivity to viruses and the frequent presence of latent agents. Besides these there are the hazards and expense connected with keeping a monkey colony and the growing concern in the world at the use of wild animals for this purpose. There has thus been an increasing need to find a replacement for monkey kidney cell culture in virus laboratories.

The use of a continuous line of cells would eliminate many of the disadvantages mentioned above. It would be easy to grow and to maintain in any quantity and its sensitivity should be reproducible from week to week and there would be no latent viruses to confuse the results. The problem is to be sure that such a line will produce an adequate cytopathic effect (CPE) to allow the detection of virus in small amounts in clinical specimens and to enable further identification procedures to be done.

With this in mind, the continuous line of African green monkey kidney cells

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(VERO) derived by Yasumura & Kawakita (1963) has been used in a series of experiments to determine whether or not it would be a suitable replacement for primary or secondary rhesus monkey kidney for the isolation and identification of a variety of human enteroviruses. The results of the experiments are given in this report.

MATERIALS AND METHODS

Tissue cultures

VERO Cells

The continuous line of African green monkey cells (*Cercopithecus aethiops*) designated VERO was obtained through Dr A. D. Macrae. Stock cultures were grown in 6 oz. flat bottles on medium 199 containing 2% foetal calf serum, 0·15% sodium bicarbonate and antibiotics. Subcultures were made twice a week when the cell sheet was confluent, by removing the cells from the glass using a mixture of 0·02% sequestric acid (EDTA) and 0·05% trypsin in Dulbecco's phosphate buffered saline solution A (P.B.S.). The cell sheets were first washed in P.B.S. then just covered with the trypsin/EDTA mixture. This was left in contact with the cells for exactly 1 min. at room temperature and then discarded. The bottle was incubated at 36–37° C. for 5 min. after which time 3 ml. of the growth medium was added, the cells were washed off the glass into this and diluted appropriately so that tubes received 1 ml. of cell suspension containing approximately 10^5 cells. The tubes were incubated in stationary racks at 36–37° C. for 48 hr. after which the medium was changed to a maintenance fluid consisting of medium 199 with 1% foetal calf serum, 0·176% sodium bicarbonate and antibiotics, and the cultures were ready for use.

Primary rhesus monkey kidney

A method based on that of Rappaport (1956) was used to trypsinize kidneys from freshly killed monkeys. The growth medium was Hanks's balanced salt solution containing 0·5% lactalbumin hydrolysate, 2% bovine serum, 0·05% sodium bicarbonate and antibiotics. Tubes were seeded with 1 ml. of suspension containing 5×10^4 cells. Maintenance fluid was medium 199 with 0·22% sodium bicarbonate and antibiotics. This replaced the growth medium one day before the cultures were used, usually seven days after trypsinization.

Virus suspensions and specimens

The viruses used were all prototype enteroviruses as listed in Table 1. The clinical specimens consisted of a variety of throat swabs, faeces, cerebro-spinal fluid and urines which were kindly supplied by the Public Health Laboratories at Leeds and Neasden, London.

Virus titrations

Four culture tubes of primary rhesus monkey kidney (p. MK) and four tubes of VERO cells were inoculated each with 0·1 ml. serial 0·5 log. dilutions. Cultures

Table 1. *Infectivity titres of enteroviruses in primary rhesus monkey kidney (p. MK) and VERO cells after three passages in p. MK and VERO cells*

		Titration of virus grown in p. MK*		Titration of virus grown in VERO cells*	
Virus	*Strain*	In p. MK	In VERO	In p. MK	In VERO
Echo 1	Farouk	7·1	6·9	6·1	6·6
Echo 2	Cornelis	6·2	7·0	5·7	6·0
Echo 3	Morrisey	6·4	7·9	6·5	7·9
Echo 4	Pesascek	5·2	< 1·0	< 1·0	< 1·0
Echo 4	du Toit	7·0	7·7	7·5	7·6
Echo 5	Noyce	7·7	7·5	7·5	8·0
Echo 6	D'Amori	6·9	8·4	7·2	8·4
Echo 7	Wallace	7·4	6·6	7·7	7·5
Echo 8	Bryson	7·2	7·0	8·1	7·5
Echo 9	Hill	6·7	6·1	7·6	7·7
Echo 11	Gregory	7·9	7·5	7·5	7·1
Echo 12	Travis	7·4	6·7	7·1	7·2
Echo 13	11–4D	5·4	6·0	5·9	6·4
Echo 14	Tow	4·5	< 1·0	6·6	7·1
Echo 15	Charleston	5·5	6·1	7·2	7·2
Echo 16	Harrington	3·9	< 1·0	< 1·0	< 1·0
Echo 17	CHHE-29	5·1	< 1·0	< 1·0	< 1·0
Echo 18	Metcalf	3·9	< 1·0	4·5	< 1·0
Echo 19	Burke	5·5	6·1	6·2	6·7
Echo 20	JV-1	4·9	1·7	4·5	5·4
Echo 21	Farina	2·9	< 1·0	< 1·0	< 1·0
Echo 22	Harris	5·4	3·2	5·5	5·6
Echo 23	Williamson	4·1	2·1	< 1·0	< 1·0
Echo 24	DeCamp	5·1	3·4	6·4	6·6
Echo 25	JV-4	6·5	4·0	6·0	6·6
Echo 26	Coronel	5·6	6·1	5·1	6·1
Echo 27	Bacon	6·9	3·2	5·2	1·9
Echo 29	JV-10	5·0	6·2	5·2	4·2
Echo 30	Bastianni	4·4	4·7	5·5	6·9
Echo 31	Caldwell	4·2	< 1·0	4·7	2·4
Echo 32	PR 10	5·6	4·1	6·1	5·9
Echo 33	Toluca-3	5·1	1·0	< 1·0	< 1·0
Echo 34	DN-19	< 1·0	< 1·0	< 1·0	< 1·0
Coxsackie A 7	Dalldorf WP 50140	6·5	6·4	7·6	6·9
Coxsackie A 9	Dalldorf 50546	7·1	< 1·0	< 1·0	< 1·0
Coxsackie B 1	P.O.	5·0	4·9	6·5	6·9
Coxsackie B 2	Ohio (Red)	5·6	6·0	7·1	7·1
Coxsackie B 3	Nancy	6·2	6·1	6·2	6·7
Coxsackie B 4	J.V.B.	6·5	6·0	6·5	6·0
Coxsackie B 5	Faulkner	4·9	3·7	7·0	6·7
Coxsackie B 6	Schmitt	5·1	6·7	5·4	7·0
Poliovirus type 1	Sabin	6·5	6·9	5·7	6·4
Poliovirus type 1	Mahoney	7·1	7·4	7·4	8·0
Poliovirus type 2	Sabin	6·0	6·1	6·3	6·4
Poliovirus type 2	YSK	6·5	6·5	6·7	7·0
Poliovirus type 3	Sabin	5·9	6·0	6·4	6·6
Poliovirus type 3	Saukett	7·0	7·1	7·1	7·2

* Expressed as log TCD 50/0·1 ml.

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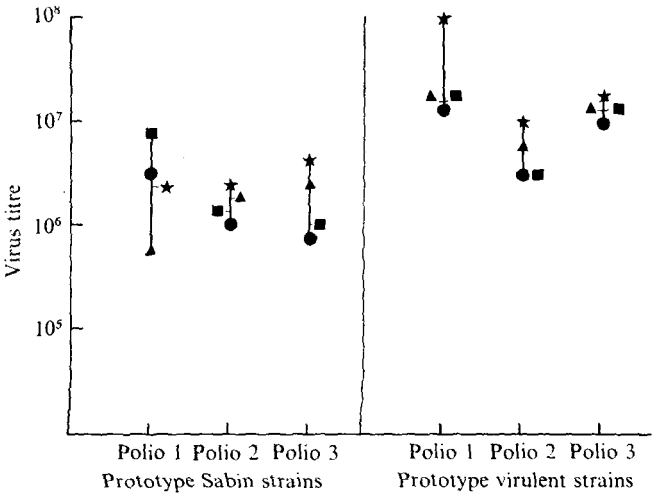

Fig. 1. Titres of the prototype polioviruses after three passages in p. MK and VERO cells. ●, MK_3 pass titrated in p. MK. ■, MK_3 pass titrated in VERO. ▲, $VERO_3$ pass titrated in p. MK. ★, $VERO_3$ pass titrated in VERO.

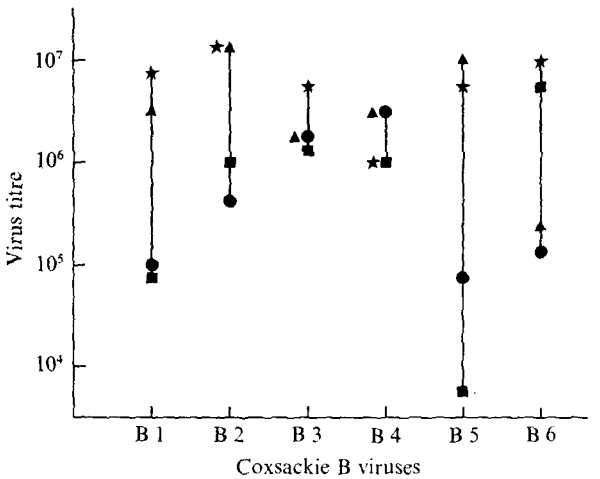

Fig. 2. Titres of the prototype Coxsackie B viruses after three passages in p. MK and VERO cells. ●, MK_3 pass titrated in p. MK. ■, MK_3 pass titrated in VERO. ▲, $VERO_3$ pass titrated in p. MK. ★, $VERO_3$ pass titrated in VERO.

were incubated at 36–37° C. and examined at intervals for CPE, the final reading being made on the sixth day. Any culture showing a distinct CPE was considered to be positive. Kärber's method (1931) was used to estimate the 50% infectivity end-points, which were recorded as log tissue culture infective dose per 0·1 ml. (log TCD50/0·1 ml.) Logarithms are expressed to the base 10.

Virus identification

The agents isolated in p. MK and VERO cells were both typed if they yielded sufficient virus growth. If either tissue did not give sufficient growth at least

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Table 2. *Isolation and identification of polioviruses and Coxsackie viruses from clinical specimens in primary monkey kidney (p. MK) and VERO cells*

Viruses isolated	No. isolated and identified in: p. MK and VERO	p. MK only	VERO only	Totals
Poliovirus (types 1 and 2)	6	—	—	6
Poliovirus (3 mixtures of types 1+2 and 2+3)	—	2	4	6
Coxsackie B viruses (types 2, 3, 4 and 5)	36	—	2	38
Coxsackie A virus (type 9)	—	13	—	13
Totals	42	15	6	63

Table 3. *Isolation and identification of echoviruses from 26 clinical specimens in primary monkey kidney (p. MK) and VERO cells*

Viruses isolated	No. isolated and identified in: p. MK and VERO	p. MK only	VERO only	Totals
Echovirus type 1	1	1	—	2
Echovirus type 4	—	10	—	10
Echovirus type 7	—	1	—	1
Echovirus type 9	—	2	—	2
Echovirus type 11	—	1	—	1
Echovirus type 14	2	2	—	4
Echovirus type 20	1	3	—	4
Echovirus type 21	1	—	—	1
Echovirus type 25	—	1	—	1
Totals	5	21	—	26

two passages were made before it was declared unsuitable for use in the identification of the agent. Neutralization tests were done as described by Hambling, Davis & Macrae (1963) using composite antiserum pools.

RESULTS

Stock prototype enteroviruses which had, with the exception of two, been propagated in p. MK were passaged three times in p. MK and VERO cells and then titrated in both cell cultures.

A cytopathic effect was produced in p. MK by 46 of the 47 viruses examined whilst 38 showed an effect in VERO cells. The CPE in all cases was typical of enteroviruses, the cells became round and refractile at first either in foci or all over the cell sheet, shrinking later and eventually falling from the glass. The following viruses failed to cause a distinct CPE in VERO cells: echovirus type 4 Pesascek, 16, 17, 18, 21, 23, 33, 34 and Coxsackie virus type A 9. A comparison of infectivity titres of the viruses tested is given in Table 1.

Out of the 47 viruses titrated, 17 (36%) gave higher titres in VERO cells than

in p. MK even though they had not been adapted to these cells. After three passages in VERO cells 26 out of 47 (55·5%) gave higher titres in VERO cells. With only three echoviruses, types 14, 20 and 31 was there evidence of the virus being adapted to VERO cells, in most of the others three passages produced no great improvement over direct titration in VERO cells from the p. MK pass material although some titres were improved by 1 or 2 log. dilutions. The titre of Coxsackie B 5 virus was increased by 3 log. dilutions. With the exception of Coxsackie B 1 virus the remaining Coxsackie B viruses and the polioviruses showed no significant differences in titre when titrated in both cell cultures (Figs. 1, 2).

The results of attempts at virus isolation from clinical material in both p. MK and VERO cells are shown in Tables 2 and 3. Altogether a virus was isolated in 89 specimens. Twenty-one specimens were negative in both tissues.

All the single type polioviruses were isolated in both cell cultures with no difficulty. However, with the three specimens containing more than one type of poliovirus the p. MK culture in each case did not detect the poliovirus type 2 whereas the VERO cells grew the type 2 and the other virus present in one case but missed the other virus in two specimens. Re-isolation from the original faecal material gave the same results. Of the 38 Coxsackie B viruses isolated all grew in VERO cells but only 36 grew in p. MK; the two which failed were both type B 5. All of the 13 Coxsackie A 9 viruses were isolated in p. MK only. Only 5 of the 26 echoviruses were isolated in both cell cultures; 21 caused CPE in p. MK only (Table 3).

All the agents were typed by neutralization tests in the cells in which they grew, those which grew in both were typed in both. The time for complete CPE to appear from the clinical material varied, taking a little longer in VERO cells than in p. MK, except for the isolation of the Coxsackie B viruses where the time was the same in both cell cultures.

DISCUSSION

A continuous cell line such as VERO has advantages over primary cell culture systems. These include ease of monolayer preparation and freedom from adventitious viruses. Such cell lines are, though, frequently found to be contaminated with mycoplasma organisms; the VERO cells used in this study were known to be contaminated with *M. orale* type I. No premature degeneration of the cell sheets was noted as a result of this contamination.

The results of titrations of the prototype enteroviruses in VERO cells and in p. MK show that, with few exceptions, VERO cells are very suitable for the laboratory manipulation of these well-adapted strains. Such procedures as the preparation of high-titred virus suspensions and the measurement of neutralizing antibody could be done effectively in these cells.

Clinical specimens were obtained from routine diagnostic virus laboratories, and so represented a cross-section of the enteroviruses present in the community during the period of this study. The results of virus isolation show that VERO

cells are not suitable for general 'field' isolation work. The failure of any of the echovirus type 4 strains to grow in VERO cells and the inability of VERO cells to grow the prototype echovirus type 4 (Pesascek) contrasted with the growth of echovirus type 4 (du Toit) provides another example of variation in susceptibility of cell cultures to prime variants of one serotype. Hsuing (1962) found that the prototype echovirus type 6 (D'Amori) failed to cause CPE in HEp 2 cells, yet out of 89 echovirus type 6 isolates reported by Pal, McQuillin & Gardner (1963) all were recovered in HEp 2 cells and only 37 in p. MK.

VERO cells may prove useful in conjunction with p. MK in isolation work as their inability to support the growth of some echoviruses could help in typing procedures by reducing the possible number of identities of a virus. VERO cells are as suitable as p. MK for the isolation of Coxsackie B viruses and may even be superior for poliovirus isolation.

The only isolation of both components of one of the three poliovirus mixtures tested occurred in VERO cells. The failure of p. MK to grow poliovirus type 2 from these mixtures may be due to the higher susceptibility of this virus to interference by adventitious viruses as compared with polioviruses types 1 and 3. Such an effect has been noted during work on polio-marker tests in this laboratory. The identity of the viruses isolated from the mixtures was confirmed by typing in the culture of isolation, and in the other culture (p. MK or VERO cells as appropriate), indicating that selection took place during isolation rather than during the typing of the viruses. In each case also, re-isolation was attempted from the original faecal extract with the same result.

Typing by neutralization tests produced complete agreement of results in both cell cultures. VERO cells had a more stable sensitivity to enteroviruses over the several subcultures of cells which were sometimes necessary during the time taken for the typing process, than that achieved by successive batches of p. MK. This made estimation of the virus dose easier in VERO cells and typing was therefore more accurate and less often frustrated by too few tissue culture doses (TCD 50) of virus. VERO cells were therefore useful for typing viruses which could be adapted to them. However, they were found to be less useful for the isolation of certain echoviruses and also Coxsackie virus type A 9.

We wish to thank the Public Health Laboratories at Leeds and Neasden, London, for making the isolation material available to us, also Miss M. Buckley for the preparation of the monkey kidney cell cultures and the technicians in training who have helped with the preparation of VERO cultures. Dr R. H. Leach of the Mycoplasma Reference Laboratory very kindly isolated and identified the *M. orale* type I from the VERO cells for us.

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