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A COMPARISON OF THE SUSCEPTIBILITY OF THREE HUMAN GUT TUMOUR-DERIVED DIFFERENTIATED EPITHELIAL CELL LINES, PRIMARY MONKEY KIDNEY CELLS AND HUMAN RHABDOMYOSARCOMA CELL LINE TO 66-PROTOTYPE STRAINS OF HUMAN ENTEROVIRUSES

J.R. PATEL, J. DANIEL and V.I. MATHAN*

The Wellcome Research Unit, Christian Medical College Hospital, Vellore 632 004, India

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The growth of prototype strains of 31 serotypes of ECHO, 3 polio, 6 Coxsackie B, 24 Coxsackie A and enterovirus serotypes 70 and 71 were tested in parallel in primary monkey kidney cells (PMK), RD cells and three gut tumour-derived differentiated epithelial cell lines (HRT-18 HT-29 in SKCO-1). All 31 serotypes of ECHO viruses grew in HT-29, 27 and SKCO-1, 5 in HRT-18, 29 in PMK and 29 in RD. There was good growth of poliovirus serotypes in all five cell types. Coxsackie B viruses grew well in all the cell lines except RD. Fifteen of the Coxsackie A viruses grew in SKCO-1, 4 in HT-29, 3 in HRT-18 and 7 in RD. Enterovirus serotypes 70 and 71 grew only in RD cells after 3 serial passages. These results showed that 2 of the gut tumour-derived cell lines, HT-29 and SKCO-1 had a markedly wider susceptibility, with comparable or wider sensitivity, for enteroviruses, than PMK and RD. While their use for field isolation from clinical samples is not yet fully established HT-29 and SKCO-1 would appear to be ideal for a variety of laboratory manipulations of the majority of enteroviruses.

enteroviruses growth in epithelial cell lines monkey kidney cells rhabdomyosarcoma cell line

INTRODUCTION

Tissue culture is still the major technique for isolation and study of the 69 recognised serotypes of human enteroviruses (Melnick and Rennick, 1980). Primate epithelial cell strains and lines and suckling mice are widely used for the isolation of enteroviruses from clinical samples and their subsequent identification, since it is well recognised that no single cell culture system is ideal (Wenner, 1972; Grist et al., 1979). A search for continuous cell lines to grow enteroviruses identified RD cells from human thabdomyosarcoma and BGM cells from African green monkey kidney as possible useful lines (Dahling et al., 1974; Davies and Phillpotts, 1974; Schmidt et al., 1975, and 1978;

* To whom correspondence should be addressed.

Wecker and Ter Meulen, 1977). We found that a variety of enteroviruses grew from faecal samples inoculated into HRT-18, a continuous differentiated epithelial cell line derived from human rectal carcinoma (Tompkins et al., 1978), during attempts to isolate coronavirus-like particles, widely prevalent in human faeces in southern India (Mathan et al., 1975; Patel et al., 1984). This paper reports a systematic study, comparing primary monkey kidney and RD cells with HRT-18 and two other gut tumour-derived differentiated epithelial cell lines, SKCO-1 and HT-29 (Fogh and Trempe, 1975), using 66 prototype strains of enteroviruses.

MATERIALS AND METHODS

Susceptibility was determined by measuring relative infectivities of virus seeds in the 5 different cell lines. In addition, some of the virus seeds were passaged 3 or 5 times and the infectivity determined to ascertain the extent of virus growth.

Virus seeds

Tissue culture grown prototype strains of 31 ECHO virus serotypes, 6 Coxsackie B virus serotypes, 3 poliovirus serotypes, enterovirus serotypes 70 and 71 and Coxsackie A virus serotypes 7, 9 and 16 were a kind gift from Dr. M.O. Roebuck, Central Public Health Laboratory, Colindale, London, U.K. (Table 1). Mouse grown prototype strains of Coxsackie A virus serotypes 1 to 24 (Table 2) were a kind gift from Dr. D.R. Gamble, Coxsackie A Virus Reference Laboratory, Public Health Laboratory, West Park Hospital, Epsom, U.K.

Tissue culture

All cell types were grown as monolayers at 37°C using media, including foetal calf serum (FCS), obtained from Flow Laboratories, Irvine, Scotland. Confluent monolayers were dispersed every 4 to 5 days using trypsin 1:250 (Difco Laboratories, Detroit, MI, U.S.A.) and disodium ethylene diamine tetraacetic acid (Sigma London Chemical Co. Ltd., Poole, Dorset, U.K.) mixture at a concentration of 0.07% and 0.03%, respectively, in Dulbecco's phosphate buffered saline A (PBSA). All tissue culture media were buffered with sodium bicarbonate and contained penicillin (100 U/ml), streptomycin (100 µg/ml) (Hindustan Antibiotics Ltd., Pune, India) and Fungizone (3 µg/ml) (ER Squibb and Sons Inc., Kingston, NJ, U.S.A.).

HRT-18 (pass 18) line of differentiated human epithelial cells derived from rectal adenocarcinoma (Tompkins et al., 1974) was a kind gift from Dr. J. LaPorte, INRA, Station de Recherches de Virologie et d'Immunologie, Thiverval-Grignon, France (LaPorte and Bobulesco, 1981). HT-29 (pass 148) and SKCO-1 (pass 34) cell lines were from human colonic tumours from two different individuals (Fogh and Trempe, 1975) and were purchased from the Memorial Sloan Kettering Institute, NY, U.S.A. According to the suppliers, no viral or other microbial contamination was present in these 2 cell lines. HT-29, SKCO-1 and RD cells were subsequently tested and found to be free

TABLE 1

Relative infectivities of enteroviruses after the first passage of seed virus in PMK, RD, SKCO-1, HT-29 and HRT-18 cells

Virus	Type	Strain	PMK	RD	SKCO-1	HT-29	HRT-18	RD pass 3 or 5 ^a
ECHO	1	Farouk	7.2	0	8.0	8.0	0	5.0 ^a
	2	Cornelius	6.7	4.0	7.2	7.7	0	7.4
	3	Morrissey	5.4	6.4	7.4	7.4	0	8.4
	4	Pesascek	7.0	6.0	7.2	8.2	0	8.2
	5	Noyce	8.0	7.4	8.0	8.4	0	9.2
	6	d'Amori	7.2	6.2	8.4	7.4	0	9.2
	7	Wallace	8.2	4.4	8.4	8.4	0	8.2
	8	Bryson	7.4	0	8.4	8.2	0	8.4
	9	Hill	5.2	0	6.4	6.2	0	8.4
	11	Gregory	7.4	6.4	8.0	7.4	0	7.4
	12	Trauis	6.0	6.4	7.0	7.1	0	9.2
	13	11-4D	5.4	5.4	7.4	7.4	0	8.2
	14	Tow	3.0	0	3.4	4.4	0	7.4 ^a
	15	Charleston	6.7	5.4	6.0	8.4	0	8.4
	16	Harrington	2.7	0	2.2	3.4	0	4.4 ^a
	17	CHHE-29	3.4	4.4	5.4	3.4	0	8.4
	18	Metcalf	0	0	0	4.4 ^b	0	6.4 ^a
	19	Burke	7.2	5.4	8.4	7.4	0	9.4
	20	JV-1	5.7	4.4	7.4	6.4	0	8.2
	21	Farina	0	0	6.2	6.2	0	8.0 ^a
	22	Harris	3.4	0	0	7.4	7.2	0 ^a
	23	Williamson	2.2	0	0	6.2	6.7	0 ^a
	24	Decamp	5.2	5.4	7.0	7.2	0	8.4
	25	JV-4	6.4	4.4	7.4	6.4	0	8.4
	26	Coronel	5.7	5.4	6.2	6.4	0	9.4
	E	27	Bacan	3.7	0	5.0	5.4	0
29		JV-10	4.7	5.0	4.2	4.4	0	7.4
30		Bastianni	4.7	5.4	5.2	4.4	0	8.2
31		Caldwell	2.7	0	0	3.4	0	6.2 ^a
32		PR-10	5.4	2.7	5.4	5.2	0	8.0
33		Toluca-3	4.4	6.4	8.2	7.2	0	8.2
Polio	1	Sabin	7.2	7.4	7.2	7.4	6.0	-
	2	Sabin	5.7	7.4	7.0	8.0	7.2	-
	3	Sabin	7.2	8.4	7.4	7.4	6.4	-
Coxsackie	B1	Conn	7.2	0	8.2	8.4	5.2	0
	B2	Ohio	6.7	0	8.2	8.4	4.2	0
	B3	Nancy	6.4	0	8.2	7.4	6.0	3.4
	B4	JVB	7.0	0	8.2	8.0	4.4	0
	B5	Faulkner	7.4	0	8.4	8.4	7.0	3.4
	B6	Schmitt	6.4	0	8.2	8.4	4.7	3.4

Results expressed as log₁₀ TCID₅₀/ml. 0, No growth; -, not tested.

^a Results of RD pass 5.

^b Repassaged after isolation in RD pass 5.

TABLE 2

Growth of Coxsackie A virus serotype in SKCO-1 and RD

Virus strain (WHO)	SKCO-1				RD	
	Seed	P1	P2	P3	Seed	P5
A1 ATCC no. VR161	0	0	0	0	0	0
A2 Fleetwood NIH Ref.	(+)	7.2	6.9	7.9	0	6.4
A3 Olsen Bayler Pool	4.4	7.4	7.7	8.4	0	8.4
A4 ATCC VR27	3.0	5.2	7.4	7.7	0	8.4
A5 ATCC VR164	0	0	2.7	3.4	0	0
A6 ATCC VR165	3.0	5.7	5.2	6.4	0	0
A7 Albany 50140	4.2	6.2	NT	7.4	+	7.4
A8 Donovan NIH Ref.	3.2	5.4	5.2	6.4	0	6.2
A9 Albany 50546	5.4	8.2	NT	9.2	0	8.2
A10 Albany 50548	2.7	5.7	7.2	8.0	+	6.4
A11 Albany 52148	0	0	0	0	0	0
A12 Texas 12 NIH Ref.	3.7	7.2	8.2	7.9	+	7.4
A13 Flores NIH Ref.	0	0	5.0	7.7	0	0
A14 G14 Bayler Pool	0	3.7	6.7	7.7	+	7.0
A15 Albany 52108	3.4	3.7	5.7	6.0	0	0
A16 G10 Bayler Pool	0	0	0	0	+	5.4
A17 G12 Bayler Pool	0	0	0	0	0	0
A18 Albany 52112	5.2	6.7	7.2	7.4	0	0
A19 ATCC no. VR177	0	0	0	0	0	0
A20 Albany 55166	0	0	0	0	0	0
A21 Kuy Kendal Baylor HA117	2.7	5.4	7.0	7.4	0	4.0
A22 ATCC no. VR182	0	0	0	0	0	0
A23 Albany 5844	0	0	0	0	0	0
A24 Albany 5720	0	0	0	0	0	0

Results expressed as \log_{10} TCID₅₀/ml. 0, No growth; +, growth of very low titre; P1, P2, P3, P5, number of passage at which titre was obtained; NT = not titrated.

All viruses not growing at P3 in SKCO-1 were carried till P5 without any further evidence of growth.

of contaminating mycoplasma. Primary monkey (*Macaca radiata*) kidney cells (PMK) were purchased from the Virology Department of this hospital and were used as secondary cultures (Grist et al., 1979). RD cells (pass 50) were purchased from Flow Laboratories, Irvine, Scotland.

HRT-18 and HT-29 cells were grown in RPMI-1640 medium supplemented with 10% FCS and 15 mM sodium bicarbonate. Confluent monolayers were subcultured at a split ratio of 1:3. SKCO-1 cells were grown in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 15% FCS, 1% (v/v) amino acids and 15 mM sodium bicarbonate and subcultured at a split ratio of 1:2. RD cells were grown in DMEM containing 10% FCS and 15 mM sodium bicarbonate at a subculture ratio of 1:2.

Serial cultivation of virus seed

Virus seeds were passaged undiluted using an inoculum of 0.1 ml per tube culture in the case of Coxsackie A viruses and others of initial apparently insufficient infectivity but otherwise cell monolayers were inoculated at an input multiplicity of infection of about 0.1 at each serial passage. All cell monolayers were grown in 1 ml medium in 16 mm × 120 mm tissue culture glass tubes. After adsorption of the inoculum for 1 h at 37°C, cultures were washed with PBSA and 1 ml of the appropriate maintenance medium (serum reduced to 2%) was added to each tube, sealed, and incubated at 37°C. Cytopathic effects (CPE) were read daily for 3 days and cultures manifesting 50% or greater CPE were frozen at -70°C until titrated for infectivity. Cultures not showing CPE were frozen on the third day after inoculation, titrated and passaged further. All cultures were titrated for infectivity on homologous cell lines and some in heterologous cell lines. Some of the virus seeds were carried through two further undiluted passages at 37°C for 3 days each and a few viruses (mainly Coxsackie A and others not manifesting CPE at third passage) were carried through 5 passages and then titrated for infectivity as indicated in the Results section.

Infectivity assays

Confluent monolayers, grown in 96-well flat bottom tissue culture plates (Sterlin Ltd., Teddington, Middlesex, U.K.), 3-4 days after seeding 2 (HT-29 and HRT-18) to 4×10^5 (SKCO-1, RD and PMK) cells in 0.15 ml medium per well were used for infectivity assays. Monolayers were washed with PBSA and inoculated with serial 10-fold dilutions of test virus suspension in Lebovitz L15 medium containing 2% FCS and antibiotics and 1% amino acid supplement (L-15-2 maintenance medium), using 20 µl inoculum per well and 4 wells per dilution and plates were incubated at 37°C for 1 h. After virus adsorption 0.1 ml of L-15-2 medium per well was added and plates were sealed (Plate sealers, Flow Laboratories) and incubated at 37°C. Cultures were examined for CPE daily for 6 days. Cultures showing progressive CPE to involve 50-100% or less (PMK) of the cell sheet on the last day of examination were recorded and used to calculate median infective dose (TCID₅₀) (Irvine and Cheeseman, 1939). On each plate 4 to 8 wells at different sites were inoculated last with maintenance medium and used to judge viral cross contamination. From the records of CPE made during titrations, wells receiving approximately 100 TCID₅₀ were chosen to obtain a reasonably comparable estimate of the progress of CPE in the 5 cell types used in this study.

Virus typing

Mixtures of equal volumes (40 µl U/vol.) each of LBM intersecting serum pools A-H containing antibodies to 43 enterovirus serotypes (Lim and Benyesh-Melnick, 1960; Schmidt et al., 1971) and test virus suspension (approximately 400 TCID₅₀), both diluted in L-15-2, were incubated at 37°C for 2 h and residual infectivity in the mixture was determined as described above but without further dilution. Virus suspensions similarly diluted and mixed with L-15-2 and incubated at 37°C for 2 h were also

inoculated into cell monolayers at serial two-fold dilutions to determine the dose of the test virus used in the typing assay. Virus seeds after growth in the various cell lines were re-identified.

RESULTS

Cytopathic effects

The cytopathic effects (CPE) in all cases was typical of enteroviruses, with cells becoming round and refractile either in foci or all over the cell sheet and subsequently degenerating. CPE in HT-29 cells occurred in all cases, except Coxsackie-A virus types 15 and 18, as distinct foci at higher dilution of virus suspensions, differing in shape and size, but with an inoculum of 100 TCID₅₀, there was almost complete destruction of the cell sheet by the second or third day of infection. Coxsackie-A virus types 15 and 18 gave rise to rounded and refractile cells all over HT-29 cell sheets. Plaque formation was also a feature in HRT-18 cells. The CPE was generally diffuse in SKCO-1, RD and PMK. Infected cells usually remained attached to the support in HT-29 and HRT-18 cells, while they usually rounded and floated into suspension in the case of SKCO-1 and RD cell monolayers.

ECHO viruses

All 31 strains of ECHO viruses grew in HT-29, 27 in SKCO-1, 5 in HRT-18, 29 in PMK and 29 in RD (Table 1). Types 18 and 21 failed to grow and produce CPE in PMK even when inoculated with undiluted virus suspension. Types 22 and 23 readily grew in HRT-18 cells and types 16, 31 and 33 grew to low titres by the third passage. The growth of most of the ECHO types in RD was poorer than in PMK, HT-29 and SKCO-1 and many grew only by the third or fifth pass (Table 1). The seed of virus type 18 did not initially grow in any of the five cell lines but at pass 5, in RD cells, it grew with a titre of 6.4. It was reconfirmed by neutralization as type 18 and the RD cell grown type 18 was re-passaged and was found to grow poorly in HT-29. The relative infectivities of virus seeds (Table 1) showed that for 13 ECHO types (ECHO 1, 2, 3, 8, 9, 12, 13, 20, 21, 24, 26, 27 and 33) HT-29 and SKCO-1 were significantly ($> 1 \log_{10}$) more sensitive than PMK cells. A further 13 serotypes grew to higher titres than in PMK in either HT-29 (4, 14, 15, 16, 22, 23, 31) or SKCO-1 (6, 11, 17, 19, 25, 30).

Polioviruses

Sabin strains of all three serotypes grew in all five cell types (Table 1).

Coxsackie-B viruses

With the exception of RD cell line in which only serotype 3, 5 and 6 grew poorly ($3.4 \log_{10}/\text{ml}$) by the fifth passage, the remaining cell types were susceptible to all six serotypes of Coxsackie-B virus (Table 1).

Coxsackie-A viruses

Of the 24 Coxsackie-A viruses, 15 grew in SKCO-1, 4 in HT-29, and 3 in HRT-18 and 11 in RD (by fifth pass) (Table 2). Tissue culture and mouse brain grown types 7, 9 and 16 grew well both in SKCO-1 and RD. The infectivity of viruses that grew in HT-29 (types 7, 9, 15, 18) and HRT-18 (types 3, 9, 21) cells was low.

Enterovirus type 70 and 71

Both these viruses grew in RD cells only after three serial passages. Type 70 in addition had low infectivity in HT-29.

DISCUSSION

The three human gut-derived epithelial cell lines tested were different in their susceptibility to defined strains of human enteroviruses (Tables 1 and 2). All serotypes of ECHO, Coxsackie-B and polioviruses grew readily and to high titres in HT-29 cells; but only 4 of 24 Coxsackie-A serotypes grew with poor to moderate titres. The growth of Coxsackie-A viruses was better in SKCO-1, 15 of 24 serotypes growing to high titre, but four ECHO serotypes did not grow. In the third gut-derived epithelial cell line, HRT-18, only 5 of 31 ECHO serotypes and only 3 of 24 Coxsackie-A viruses grew. These differences between the three gut tumour-derived epithelial cell lines might be accounted for by differing virus receptor expression and other metabolic differences between the cells. Furthermore, HRT-18 is heavily contaminated with several types of mycoplasma which might also account for the poor growth of enteroviruses. The other cell lines were free of mycoplasma. A variety of respiratory viruses (Coronavirus serotypes OC43 and 229E, Rhinovirus types 2 and 9, and respiratory syncytial virus) were subsequently found not to produce CPE in HT-29 monolayers in rolled cultures at 33°C (Patel, Al-Nakib and Tyrrell, unpubl. data). It therefore appears likely that the gut tumour-derived epithelial cells are particularly suited only for viruses that infect the gastrointestinal tract.

RD (Schmidt et al., 1975; Wecker and ter Meulen, 1977), Vero (Davies and Phillpotts, 1974; Wecker and ter Meulen, 1977) and BGM (Dahling et al., 1974; Schmidt et al., 1978) cell lines have been reported to be susceptible to a variety of enteroviruses. It is difficult to make direct comparisons of results obtained in different studies, since virus strain and cell batch related factors may affect the growth patterns (Schmidt et al., 1975). The results presented in Tables 1 and 2 show that the growth of ECHO and Coxsackie-B virus serotypes was better in one or both of the two intestinal tumour-derived cell lines, HT-29 and SKCO-1, compared to RD cells. Fifteen of the 24 mouse brain grown Coxsackie-A viruses also grew to high titres in SKCO-1 and their growth was much better than in RD cells in which several of these viruses are reported to grow well (Schmidt et al., 1975).

These results show that intestinal tumour-derived epithelial cells in continuous culture support the growth to a high titre of a wide variety of prototype strains of

enteroviruses and are useful for laboratory manipulations including neutralisation, detection of antibodies and for growing virus in quantity. However, the use of these cells for the field isolation of enteroviruses has to be established. In preliminary studies, 34 CPE producing agents (ECHO 7-6, ECHO 30-4, Polio 2-8, Coxsackie B6-2 and untyped 14) were isolated from 72 stool samples obtained from 39 individuals belonging to ten families from a village affected by an epidemic of diarrhoea (unpubl. data). These results are suggestive that in the field isolation of enteroviruses SKCO-1 and HT-29 cells is likely to be as useful as it has been shown to be for various laboratory procedures in the present study. A large number of intestinal neoplasm-derived continuous epithelial cell lines are now available (Fogh et al., 1977) and it would appear profitable to investigate them to detect cells which may support growth of a wider variety of enteroviruses.

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