Isolation and Identification of Enteroviruses From Faecal Samples in a Differentiated Epithelial Cell Line (HRT-18) Derived From Human Rectal Carcinoma

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A human rectal carcinoma-derived differentiated epithelial cell line, HRT-18, was inoculated with faecal samples in an attempt to grow coronavirus-like particles (CVLP), which are widely prevalent in human stools in southern India. While CVLP did not grow in this cell line, a variety of enteroviruses were isolated from 48 of the 114 stool samples from healthy controls and patients with diarrhoea. The results suggest that human gastrointestinal tumour-derived differentiated epithelial cells in continuous culture may be useful for the primary isolation of enteroviruses and merit further study.

Key words: enteroviruses, tissue culture, HRT-18 cells

INTRODUCTION

A differentiated human rectal carcinoma-derived epithelial cell line, HRT-18 [Tompkins et al, 1974] was inoculated with human faecal samples in an attempt to grow coronavirus-like particles (CVLP), which are widely prevalent in the stools of controls and patients with tropical sprue in southern India [Mathan et al, 1975]. Several enteric coronaviruses grow well in HRT-18 cells [Laporte and Bobulesco, 1981] but CVLP did not grow. However, a variety of enteroviruses were isolated in this cell line from 48 of the 114 stool samples tested, and the results presented here suggest that differentiated epithelial cells derived from intestinal tumours may be useful additional cell lines for the isolation of enteric viruses. It was also possible to identify rapidly the isolates by neutralisation in a microtitre system with HRT-18 cells.

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MATERIALS AND METHODS

Faecal Samples

Single stool samples were collected in 1982 from three groups of subjects:

1) Samples were collected from 20 hospitalised adults, seven of whom had acute diarrhoea, six with chronic diarrhoea (tropical sprue) [Baker and Mathan, 1971], and seven without gastrointestinal illness.

2) Village R, 25 km to the south of Vellore, had been affected by an epidemic of tropical sprue [Mathan and Baker, 1971] in 1975 and was kept under surveillance. A further outbreak of acute diarrhoea occurred in this village in March 1982, and samples were collected from patients and appropriate controls on April 3, July 13, and July 20. These samples were from seven preschool children (age below 5 years), 19 school children, and 18 adults. The seven adults with acute diarrhoea in group 1 were from this village. The details of this epidemic will be published elsewhere.

3) Faecal samples were also obtained from ten families who belonged to a stratified random sample of a village 20 km to the south of Vellore. These healthy controls consisted of seven preschool children, 11 school children, and 32 adolescents and adults.

Approximately 20% (w/v) suspensions of faeces were prepared in RPMI 1640 (Flow Laboratories, Irvine, UK) containing 0.5% bovine serum albumin (Armour Fraction V, Sigma London Chemical Co., Ltd., Poole, UK) 200 units/ml penicillin, 200 µg/ml streptomycin, and 35 mM bicarbonate (RPMI-BSA). Suspensions were centrifuged at 4,000g for 30 minutes and the supernatant of each, after a further centrifugation, was divided into three aliquots, one used for virus isolation and the other two aliquots stored at -70°C .

Cell Culture

All tissue culture media, including fetal calf serum (FCS), were obtained from Flow Laboratories. Tissue culture plates (96-well) with lids used in microneutralisation tests were from Sterilin Ltd. (Teddington, UK). HRT-18 cells provided by Dr. Jacques Laporte (INRA, Station de Recherches de Virologie et d'Immunologie Thiverval-Grignon, France) were grown in 20-oz glass medical flat bottles at 37°C in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, 10 µg/ml streptomycin, and 15 mM sodium bicarbonate (RPMI 10). After 4–5 days cells from confluent monolayers were subcultured at a ratio of 1:3, after dispersion with Difco-250 trypsin (0.07%) and disodium versene (0.03%) mixture in Dulbecco's phosphate-buffered saline, pH 7.3 (PBS).

Standard Method for Virus Isolation

Confluent monolayers, obtained 3 days after seeding approximately $1-2 \times 10^6$ HRT-18 cells in 6 ml RPMI 10 per 2-oz bottle, were washed with PBS and inoculated with 0.5 ml of freshly prepared stool suspension, which was allowed to adsorb for 1 hour at 37°C . The monolayers were then washed and incubated at 37°C adding maintenance medium, RPMI 1640 containing 2% FCS, 30 mM sodium bicarbonate, and antibiotics (RPMI 2). Cultures were examined daily for cytopathic effect (CPE) and frozen at -70°C for further passage after 3 days. Two further passages were carried out as described above. Monolayers inoculated with RPMI 2 were included as controls.

Isolation in the Presence of Trypsin

Clarified stool suspension (0.5 ml) in RPMI-BSA was incubated at 37°C for 20 minutes with crystalline III Trypsin (Sigma London Chemical Co., Ltd.) at a final concentration of 3 µg/ml. This was inoculated onto confluent, washed HRT-18 monolayers, allowed to adsorb, and maintained in serum-free RPMI 1640 supplemented with 0.5% BSA, 0.5% yeast extract (Difco Laboratories, Detroit, MI), 40 mM sodium bicarbonate, antibiotics, and 1 µg/ml crystalline trypsin (RPMI-T). HRT-18 monolayers inoculated with RPMI-BSA containing 3 µg/ml trypsin and maintained on RPMI-T were used as controls. Isolation in the presence of trypsin was attempted only for stool samples that did not yield isolates in the conventional culture. The concentration of trypsin was determined in experiments with uninfected tissue cultures as the maximum amount not visibly damaging the cell layers.

Preparation of Monolayers in 96-Well Microtitre Plates

About 2×10^5 HRT-18 cells suspended in 0.1 ml Leibovitz L-15 medium supplemented with 15% FCS, antibiotics, and 1% Flow's amino acid were added to each well of 96-well microtitre plates. Covered plates were incubated at 37°C in a humidified incubator for 3 days to obtain confluent monolayers.

Infectivity

Washed monolayers in 96-well microtitre plates were inoculated with serial tenfold dilutions of third-passage virus isolates using 20 µl per well and 4 wells per dilution. Diluent was Leibovitz L-15 medium with L-glutamine containing 0.5% BSA (L-15-BSA). After 1 hour adsorption at 37°C, 0.1 ml L-15 medium containing 2% FCS (L-15-2) was added to each well and the plates were incubated at 37°C as before. Several wells inoculated with L-15-BSA were used as controls. CPE was read daily for 4 days and median infective dose (TCID₅₀) was determined [Irwin and Cheeseman, 1939].

Nucleic Acid Typing

Inhibition of growth of viruses in the microtitre tray system of 2 log₁₀ or greater by 10⁻⁴ M 5-bromo-2-deoxyuridine (2 BDU, Sigma London Chemical Co., Ltd.) was taken to indicate DNA viruses. Cultures were maintained in L-15-BSA medium supplemented with 1% Flow's amino acid preparation and 0.5% yeast extract. Titre differences of 0.5 log₁₀ or less were considered expected assay variation. An isolate of adenovirus was used as a DNA virus control.

Identification

Third-passage HRT-18 isolates were identified by neutralisation using LBM serum pools in microtitre tray cultures [Lim and Benyesh-Melnick, 1960; Schmidt et al, 1971]. Twenty-five-microlitres of each serum pool diluted tenfold in L-15-BSA and an equal volume of virus suspension diluted in L-15-BSA to contain about 3 log₁₀ TCID₅₀/25 µl were incubated at 37°C for 1.5 hours. Each mixture was inoculated into two wells, 20 µl per well and adsorbed for 1 hour at 37°C. L-15-2 (0.1 ml) was then added to each well and plates were incubated at 37°C as before. Cultures were read for CPE daily for 3 days.

Electron Microscopy

About 10% w/v faecal suspensions in PBS were centrifuged at 10,000g for 30 minutes and the supernatant was centrifuged at 90,000g for 90 minutes. The pellet was resuspended in distilled water and examined in a Philips EM 201C electron microscope after negative staining on Carbon Parlodion-coated grids [Mathan et al, 1975]. Tissue culture fluid was also examined to detect CVLP and to study virus morphology.

RESULTS

Electron Microscopic Examination of Stool Samples

Fringed pleomorphic virus-like particles resembling coronaviruses (CVLP) were seen in 96 of the 114 stool samples. In addition, small, round viruses resembling enteroviruses were present in four stool samples that were obtained from an infant, a 9-year-old boy, and two adult males. Enteroviruses were isolated from the former two, but these need not necessarily be the same viruses as those seen in the stool sample.

Virus Isolations in HRT-18 Cell Monolayers

Fifty-one cytopathic isolates were obtained from the 114 stool samples. Forty-three isolations were obtained in standard HRT-18 monolayers and an additional eight isolates were obtained in the presence of trypsin from the 71 samples, which were negative in the standard system. Forty-eight of the isolates that contained RNA were confirmed to be enteroviruses by neutralisation tests with LBM pools. The cytopathic effect (CPE) of the enteroviruses was characteristic with the cells becoming round and refractile initially in isolated foci or occasionally diffusely, the change progressing to involve 60–90% of the monolayer within 48 hours. Another isolate from an 8-year-old girl contained RNA but was not neutralised by the reference antisera, although it had typical enterovirus CPE.

The growth of two other isolates was significantly reduced by 2 BDU; they were not neutralised by the LBM pools of sera and they had typical adenovirus morphology. Adenovirus CPE in HRT-18 was distinctly different from that of enteroviruses. Initially there were clusters of clumped, swollen granular cells in foci that progressed to involve all the monolayer in 3 to 4 days.

The enterovirus isolates were ECHO serotypes 3, 22, 23, and 31, poliovirus type 2, and Coxsackie A7 (Table I). The additional isolates in the presence of trypsin did not belong to a single serotype. Over two thirds of the enteroviruses isolated produced CPE by the second passage (Table I) but the two adenoviruses and 13 enteroviruses were isolated only at the third passage. Infectivity titres determined for third-passage isolates were over 8 log₁₀ TCID₅₀/ml for all except one ECHO 22 isolated with trypsin, poliovirus type 2 (eight isolates), Coxsackie A7, and the two adenovirus isolates (Table I).

After the completion of the study, ten original stool samples (ECHO 22 [four], ECHO 23 [four], and Polio 2 [two]) were coded and passaged through HRT-18 cells, and CPE-producing agents were reidentified. This confirmed that there was no cross contamination of tissue culture.

Examination of tissue culture fluids with the electron microscope showed that enteroviruses isolated by conventional tissue culture had no obvious surface features

TABLE I. Virus Isolations in HRT-18

Virus serotypes	Total isolates	Isolated in		Isolated at passage			3rd passage titre log ₁₀ TCID ₅₀ /ml
		Conventional	Trypsin	1st	2nd	3rd	
ECHO 3	1	—	1	—	1	—	8.2
ECHO 22	20	19	1	4	15	1	8.3 to 9.5
ECHO 23	17	17	—	6	6	5	8.5 to 9.5
ECHO 31	1	—	1	1	—	—	8.7
Polio 2	8	5	3	—	2	6	4.8 to 5.6
Coxsackie A7	1	1	—	—	—	1	7.4
Untyped	1	1	—	—	1	—	9.3
Adeno	2	1	1	—	—	2	6.5; 7.4

TABLE II. Virus Isolations in Different Age Groups and Different Subject Groups

Virus serotype	No. of isolates	Age group (years)				Area groups		
		0-4.9	5-11.9	12-18	18+	Ward	Vill. R	Vill. Ka
		Preschool (N = 14)	School (N = 30)	Adolescents (N = 7)	Adults (N = 63)	(N = 20)	(N = 44)	(N = 50)
ECHO 3	1	—	1	—	—	—	1	—
ECHO 22	20	5	2	—	13	5	14	1
ECHO 23	17	3	5	1	8	3	8	6
ECHO 31	1	—	1	—	—	—	1	—
Polio 2	8	1	1	2	4	—	2	6
Coxsackie A7	1	—	—	—	1	—	—	1
Untyped	1	—	1	—	—	—	—	1
Adeno	2	1	1	—	—	—	1	1
Total	51	10	12	3	26	8	27	16

and were mainly full particles. In contrast, the isolates obtained in the presence of trypsin tended to be ovoid in shape and many particles were empty. CVLPs were not detected by electron microscopy in any of the tissue culture fluids.

Prevalence of Enteroviruses

Viruses were isolated from all age groups and in subjects from all three sources (Table II). There was an epidemic of diarrhoea in village R particularly affecting the adults, and ECHO 22 was mainly isolated from these patients. The epidemiological and clinical details will be correlated to viral isolations and published elsewhere. Even discounting the isolation of ECHO 22 from patients with acute episodes of diarrhoea, there was a high rate of isolation of viruses especially ECHO 23 and Polio 2 from the adults (14/50 excluding adults with acute diarrhoea) (Table II).

DISCUSSION

The primary aim of this study was to attempt to grow CVLP [Mathan et al, 1975] in vitro in HRT-18, a differentiated, rectal carcinoma-derived epithelial cell

line known to support the growth of bovine enteric coronaviruses [Laporte and Bobulesco, 1981]. The addition of trypsin to the culture system was prompted by the report of its potentiating effect on the growth of several fastidious enteric viruses and in particular that of bovine coronavirus [Dea et al, 1980; Storz et al, 1981; Toth, 1982]. Although CVLP failed to grow in this tissue culture system, a variety of enteroviruses were isolated (48/114) with a frequency of over 25%, even in adults without diarrhoea (Table II). This high rate of isolation of enteroviruses suggests a widespread prevalence in the rural population in southern India. An enterovirus prevalence of 25% was reported from Vellore [Feldman et al, 1970] after inoculation of rectal swabs from preschool children in primary monkey kidney cells (PMK) and suckling mouse brain (SMB). The rate of enterovirus isolation was of the order of 7 to 13% from adults in studies of epidemics of tropical sprue using PMK and SMB [Mathan and Baker, 1971]. The rate of isolation of enteroviruses especially from adults, using HRT-18, a gut-derived continuous epithelial cell line, was higher than this. The predominance of ECHO 22 in patients with diarrhoea and ECHO 23 in controls suggests that epidemics due to these two viruses were occurring at the time of faecal sampling. A high rate of asymptomatic infection with enteric bacterial pathogens has been shown in this population [Rajan and Mathan, 1982] and the results of this study suggest that a similar situation exists also for enteroviruses.

Tissue culture is the major technique for the field isolation and identification of enteroviruses, of which 68 serotypes are now recognised [Melnick and Rennick, 1980]. PMK and one or more of primary or semicontinuous cell lines of human origin and inoculations of suckling mice are necessary to isolate enteroviruses because of the limited susceptibility of individual cell systems. Since there are several well-recognised drawbacks associated with the use of PMK cells [Davis and Philippotts, 1974], a replacement was sought. Vero cells have been shown to be useful for the isolation of Cocksackie B viruses and Polioviruses. Although 24 of 33 PMK-adapted ECHO virus strains grow in Vero cells, the cell line was not considered suitable for primary field isolation of ECHO viruses [Davis and Philippotts, 1974]. A continuous cell line derived from human rhabdomyosarcoma (RD cells) was reported to support the growth of 14 of 24 Cocksackie A viruses [Schmidt et al, 1975]. RD cells were subsequently found to be better than Vero cells for isolating and growing ECHO viruses and polioviruses [Wecker and ter Meulen, 1977]. Although it has been suggested that human foetal intestinal organ culture may be useful for the isolation of fastidious enteric viruses [Patterson et al, 1975; Caul and Eggleston, 1977] and several tissue culture-adapted viruses have been successfully grown in intestinal organ cultures [Stenhouse, 1970], this would appear to be impractical for large scale field studies.

Continuous cell lines clearly offer the advantages of lower cost, easy and regular supply, greater reproducibility of results, and lack of unexpected confusion caused by the presence of latent viruses. One of the primary sites of replication of enteroviruses is the gastrointestinal mucosa, although it is not clear whether this occurs in epithelial cells, lymphoid cells, or both [Wenner, 1972]. Several enteroviruses were isolated from stool, in the rectal tumour-derived differentiated epithelial cell line HRT-18, with characteristic CPE and grew rapidly to high titres. HRT-18 monolayers in 96-well microtitre trays were particularly useful for nucleic acid typing, infectivity titration, and virus identification by neutralisation. The results reported here suggest that human gut tumour-derived differentiated epithelial cells like HRT-18 and several

others now available are likely to be a useful alternative for enterovirus isolation and merit a detailed study.

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REFERENCES

- Baker SJ, Mathan VI (1971): Tropical sprue in southern India. In "Tropical Sprue and Megaloblastic Anaemia." A Wellcome Trust Collaborative Study. London: Churchill Livingstone, pp 189–260.
- Caul EO, Egglestone SI (1977): Further studies on human enteric coronaviruses. *Archives of Virology* 54:107–117.
- Davis PM, Phillpotts RJ (1974): Susceptibility of the VERO line of African green monkey kidney cells to human enteroviruses. *Journal of Hygiene [London]* 72:23–30.
- Dea S, Roy RS, Begin ME (1980): Bovine coronavirus isolation and cultivation in continuous cell lines. *American Journal of Veterinary Research* 41:30–38.
- Feldman RA, Christopher S, George S, Kamath KR, John TJ (1970): Infection and disease in a group of south Indian families. III. Virological methods and a report of the frequency of enteroviral infection in preschool children. *American Journal of Epidemiology* 92:357–366.
- Irwin JO, Cheeseman EA (1939): On an approximate method of determining the median effective dose and its error in the case of a quanta response. *Journal of Hygiene [London]* 39:574–580.
- Laporte J, Bobulesco P (1981): Growth of human and canine enteric coronaviruses in a highly susceptible cell line: HRT-18. *Perspectives in Virology* 9:189–193.
- Lim KA, Benyesh-Melnick M (1960): Typing of viruses by combination of antiserum pools. Application to typing of enteroviruses (Coxsackie and ECHO). *Journal of Immunology* 84:309–317.
- Mathan VI, Baker SJ (1971): The epidemiology of tropical sprue. In "Tropical Sprue and Megaloblastic Anaemia." A Wellcome Trust Collaborative Study. London: Churchill Livingstone, pp 159–188.
- Mathan M, Mathan VI, Swaminathan SP, Yesudoss S, Baker SJ (1975): Pleomorphic virus-like particles in human feces. *Lancet* 1:1068–1069.
- Melnick JL, Rennick V (1980): Infectivity titres of enterovirus as found in human stool. *Journal of Medical Virology* 5:205–220.
- Patterson S, Parry J, Matthews THJ, Dourmashin RR, Tyrrell DAJ, Whitehead RG, Rowland MGM (1975): Viruses and Gastroenteritis. *Lancet* 3:451.
- Rajan DP, Mathan VI (1982): Prevalence of *Campylobacter fetus* subsp. *jejuni* in healthy populations in southern India. *Journal of Clinical Microbiology* 15:749–751.
- Schmidt NJ, Ho HM, Lennette EH (1975): Propagation and isolation of group A Coxsackie viruses in RD cells. *Journal of Clinical Microbiology* 2:183–185.
- Schmidt NJ, Melnick JL, Wenner HA, Ho HM, Burkhardt MA (1971): Evaluation of enterovirus immune horse serum pools for identification of virus field strains. *Bulletin of the World Health Organization* 45:317–330.
- Stenhouse AC (1970): Virus multiplication in organ cultures of human embryo small intestine. *Journal of General Virology* 8:235–239.
- Storz J, Rott R, Kaluza G (1981): Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. *Infection and Immunity* 31:1214–1222.
- Tompkins WAF, Watrach AM, Schmale JD, Schultz RM, Harris JA (1974): Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum. *Journal of the National Cancer Institute* 52:1101–1110.
- Toth TE (1982): Trypsin enhanced replication of neonatal calf diarrhea coronavirus in bovine embryonic lung cell. *American Journal of Veterinary Research* 43:967–972.
- Wecker I, ter Meulen V (1977): RD cells in the laboratory diagnosis of enteroviruses. *Medical Microbiology and Immunology* 163:233–240.
- Wenner HA (1972): The enterovirus. *American Journal of Clinical Pathology* 57:751–761.