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Enteric Virus Isolation in Different Cell Cultures *

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The need for establishing an optimum tissue culture cell line for isolation of enteric viruses prompted a comparative study of cell lines for the isolation of enteric viruses from rectal swab specimens. The viruses isolated included all three types of poliovirus, 12 types of echovirus, seven types of group A and two of group B coxsackievirus, 24 strains of adenovirus, and eight untyped agents which probably represent new enteroviruses. One hundred positive isolations were made from 387 rectal swab specimens; 86 were obtained in human kidney cultures. This was almost twice the number obtained with monkey kidney and more than one-and-a-half times that obtained with human lung cells (WI-38). A diploid human epithelial cell line (Attleson) was tested and was found to be inferior to both monkey kidney and WI-38 cells for enteric viruses. Human kidney cells were particularly useful for group A coxsackieviruses and adenoviruses.

With the advent of tissue culture, a number of viruses have been isolated from the human alimentary tract. As a consequence, our knowledge of these agents and of the infections they cause has greatly increased. These viruses are of public health importance for they are responsible for outbreaks of diseases, both minor and major, such as poliomyelitis, aseptic meningitis, respiratory illnesses, exanthems and at times diarrhoea of infants. The presence and incidence of these viruses in a community can be determined by epidemiological surveys. Ideally the system employed should be one which permits the recovery of as many viral agents as are present in the specimens tested. This ideal can be approached by using simultaneously a number of different cell cultures (and infant mice) but this is often not convenient or feasible in any one laboratory. More realistic would be the selection of one or two cell types with the broadest spectrum of susceptibility to these viruses.

Many investigators have reported the susceptibility of a variety of cell cultures to human enteroviruses (Hsiung, 1962; Hsiung & Melnick, 1957, 1958; Kalter et al., 1962; Marchetti & Gelfand, 1963; Wenner, 1962). Often, the tests for susceptibility were carried out with prototypic and established strains of viruses with results not necessarily applicable in primary isolations. Kelly & Sanderson (1962) compared the sensitivities of four kinds of tissue cultures and recommended the use of both monkey kidney and human amnion cell cultures for the isolation of enteroviruses. Podoplekin & Idina (1963) reported that for the isolation of echoviruses, cultures of human embryonic fibroblast cells were more sensitive than cultures of a stable line of human amnion cells. The need to establish the optimal cell cultures seems indicated.

In conjunction with studies being carried out on infantile diarrhoea, different cell cultures were used for the isolation of enteric viruses from rectal swab specimens. This paper reports the comparative findings with monkey and human cultures and stresses the superiority of the human kidney cell culture.

MATERIALS AND METHODS

Specimens

Rectal swab specimens collected during the course of studies of infantile diarrhoea were used for this investigation. They were obtained from infants between the ages of 2 weeks and 2 years attending one of the following hospitals:

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(*a*) The Children's Hospital, Mexico City, during the months of February and March 1963. Of 113 specimens obtained, 70 were from patients with diarrhoea and 43 from non-diarrhoeal subjects. These specimens were made available to us by Dr Manuel Ramos Alvarez.

(*b*) The paediatric unit of the City-County Hospitals (Jefferson Davis and Ben Taub) in Houston, Texas, during a period of one year from November 1962 through October 1963. Of 274 specimens obtained, 139 were collected from patients with diarrhoea and 135 from a control group with no diarrhoeal disease.

Immediately after collection, the rectal swabs for viral studies were extracted in Hanks' balanced salt solution containing 1000 units of penicillin per ml or 1000 μg of streptomycin per ml and stored at -20°C. Prior to testing, the specimens were thawed and spun down at 3000 rev/min for 15 minutes in a cold centrifuge. The clarified supernatant was used as inoculum in 0.1-ml amounts into three tube cultures for each cell type or on some occasions in 0.15-ml amounts into two tube cultures.

Cells

All the specimens were tested in three principal cell cultures—namely, monkey kidney, human kidney and human lung (both the WI 38 and local diploid strains). In addition, the susceptibility of a human diploid epithelial cell line (Attleson) was studied.

Monkey kidney (MK) cells. Kidney cells from immature rhesus monkeys were grown and maintained in Melnick's lactalbumin medium as described by Wallis & Melnick (1962).

Human kidney (HK) cells. Primary cultures of human kidneys obtained from foetuses and young infants were prepared by a procedure similar to that for MK cells. These were grown in Melnick's medium with 10% foetal bovine serum and maintained in Eagle's medium with 5% calf serum and 0.22% $NaHCO_3$.

Human lung cells, WI-38. This cell strain was kindly supplied by Dr Leonard Hayflick, Wistar Institute, Philadelphia. Tube cultures at passages ranging from the 24th to the 35th were prepared by the method described by Hayflick & Moorhead (1961) and were used in all the experiments.

Human embryonic lung cells, Baylor. Local strains of human diploid fibroblasts were prepared from human embryonic lungs by a method similar to the one used for MK and were maintained in serial cultivation in 16-ounce (ca 0.5-litre) prescription bottles. When required, these were trypsinized to prepare tube cultures. These cells were not used beyond 10 passages from the primary cultivation. The maintenance medium for these cells was the same as for human kidney.

Human diploid epithelial (Attleson) cells. During the course of the present study the Attleson cell line was made available to this laboratory by Dr David Yohn of Roswell Park Memorial Institute, Buffalo, N.Y. The cell line had been derived from the chest fluid of a male patient with carcinoma of the nasal septum. It was maintained in continuous passage by a method similar to the one used for maintaining WI-38 cells and has retained a diploid status, even though it is epithelioid in character.

Isolation

Where possible, each specimen was inoculated into cultures of two or more cell types on the same day. All cultures were incubated stationary at 37°C and readings for cytopathogenic effect (CPE) were made periodically. The harvests from tubes showing CPE of about 75% of the cells were pooled after overnight storage at -20°C. Other cultures were kept for 7-14 days and discarded when the control tubes showed degeneration. For all specimens harvested, an additional passage was made in homologous cell culture to confirm isolation and in an attempt to raise the titre of the virus before typing.

Identification

Typing of " second-passage " isolates was carried out by the CPE neutralization test in the cell system in which the isolation was made. Equal volumes of virus (diluted 10^{-2} or 10^{-3} without prior titration) and antisera were incubated at 37°C for one hour, and 0.2 ml of the mixture was inoculated into each of two tube cultures. Each isolate was titrated in the same test and the typing repeated if there was an indication that too much virus had been used. The isolates were first screened with antisera for poliovirus types 1, 2 and 3 singly and as a mixture of the three sera. They were then tested with antiserum pools (Lim & Benyesh-Melnick, 1960; Schmidt et al., 1961). One set of 13 pools contained immune sera to the coxsackieviruses A7, A9, A14, and A16, the coxsackieviruses B1-B6, and the majority of the known echoviruses. These

TABLE 1
VIRUS ISOLATIONS IN DIFFERENT CELL CULTURES

Source	No. of specimens		Cell culture		
	Tested	Positive	Monkey kidney	Human kidney	Human lung, WI-38
Mexico City	113	31	10	31	7
Houston	274	69	36	55	43
Total	387	100	46	86	50

pools were specially prepared for identifying the viruses which would grow in MK cells. Another set of eight pools was used for typing members of the coxsackie A group which grow in tissue culture, as well as echovirus 21 (which grows in human but not in monkey cells). Isolates showing CPE suggestive of adenoviruses were confirmed by the complement-fixation test for the adenovirus group antigen and in some cases identified by the neutralization test with antisera to adenoviruses types 1-8.

RESULTS

Virus isolations in different cell cultures

The results of virus isolations from rectal swabs of infants in three different cell cultures are summarized in Table 1. Of a total of 387 specimens tested, 100 gave positive isolations. The highest number of isolations, 86 (86%), was made in HK cultures and this was almost twice the number in MK cells and more than one-and-a-half times that in WI-38 cells. These ratios alter somewhat when the isolations from Mexico City and Houston are considered separately.

In regard to the specimens from Mexico City, all the 31 isolates were obtained with HK cells, 10 (32%) with MK and only 7 (23%) with WI-38 cells. The HK isolates included all those obtained with MK and with WI-38 cells. There were 5 isolates which grew in both MK and WI-38 cells, 5 others which grew in MK but not in WI-38 cells and 2 which grew in WI-38 but not in MK cells. The Houston specimens yielded 69 viruses, of which 55 (80%) were isolated in HK, 36 (52%) in MK and 43 (62%) in WI-38 cells. Only 19 isolates grew in all three cell cultures, 16 in HK and WI-38, 6 in MK and HK, 5 in MK and WI-38, 14 in HK only, 6 in MK only, and 3 in WI-38 only. HK cultures again gave the highest isolation rate, with WI-38 next and slightly better than MK cells.

During the early stage of this study, local diploid fibroblast cultures were used in parallel with the other three cell cultures for the specimens from Mexico City. Four positive isolations resulted; three of these, however, were also made in WI-38 and one in HK cells. There did not appear to be any advantage, therefore, in using the local lung cells and they were omitted when the Houston specimens were tested.

Sensitivity of cell culture to viruses isolated

The types and frequency of the viruses isolated in each of the cell cultures are shown in Table 2. A total of 101 viruses were recovered from 100 positive specimens, one of the Mexican specimens having yielded two viral agents (poliovirus 3 and echo 1) in HK.

TABLE 2
SENSITIVITY OF CELL CULTURE TO VIRUSES ISOLATED

Virus		Cell culture		
Type	No. isolated	Monkey kidney	Human kidney	WI-38
Polio	15	11	11	6
Echo	31	22	26	22
Coxsackie A	20	9	16	16
Coxsackie B	3	1	3	0
Adeno	24	2	24	1
Untyped	8	1	7	5
Total	101 [a]	46	87	50

[a] One specimen yielded two viruses; thus 101 viruses were obtained from 100 positive specimens.

HK gave as good results as MK for isolation of polioviruses but WI-38 was much less sensitive. Echoviruses grew well in all three cell types, with HK having a slight advantage. Both HK and WI-38 were superior to MK for coxsackie A viruses which, except for types A9, A7, A14 and A16, do not generally propagate in MK cells. HK was particularly useful for adenoviruses compared to the other two cell cultures as all the adenovirus isolations were made in it. Of the eight isolates as yet unidentified, HK cultures yielded seven and WI-38 yielded five.

The superiority of HK cells with regard to their sensitivity to a wider range of viral agents was clearly shown. WI-38 appeared as sensitive as MK for isolations of echoviruses and superior for coxsackie A viruses, although less sensitive for polioviruses.

Types of viruses isolated

The frequency of the types of viruses isolated in each of the different cell cultures is given in Table 3. All three types of poliovirus were isolated. One poliovirus type 2 was isolated from a Mexican child and eight were isolated from Houston children belonging to the control group. The latter could well be vaccine strains, for oral vaccine had been fed in Houston prior to the collections. It was interesting to note also that three of these were isolated only in MK but not in HK or WI-38 cells. The lung cells were relatively less susceptible to polioviruses than MK or HK cells, although one of the isolations was made only in WI-38 cells and not in MK or HK cells. The four polioviruses type 3 were all from the Mexican specimens.

Twelve types of echovirus were represented in the total of 31 isolations. No distinction was made between echovirus types 1 and 8 because of their close antigenic relationship. More types of echovirus were isolated in HK than in MK or WI-38. The Mexican specimens accounted for two echovirus 1, two echovirus 6 and one each of echovirus 12 and echovirus 21. The remaining echoviruses came from Houston and the predominance of echovirus 1 in this community should be noted.

The 20 group A coxsackieviruses comprised seven types. Both HK and WI-38 supported the growth of all these types but MK yielded only two of the types, namely, A9 and A18. Coxsackie A9 strains, however, were recovered more successfully in MK than in HK and WI-38. Group B coxsackieviruses, two of type B1 and another of type B3, were found in only three specimens.

TABLE 3
TYPES OF VIRUSES ISOLATED

Virus			Cell culture		
	Type	No. isolated	Monkey kidney	Human kidney	WI-38
Polio	1	2	1	2	1
	2	9	8	5	4
	3	4	2	4 [a]	1
Echo	1	13	12	12 [a]	12
	2	1	1	1	1
	3	1	1	1	1
	6	2	1	2	0
	7	1	1	1	1
	11	1	1	1	0
	12	1	0	1	0
	14	4	4	2	3
	21	3	0	2	2
	22	1	0	1	0
	29	2	0	2	2
	31	1	1	0	0
Coxsackie	A9	8	8	4	6
	A13	2	0	2	2
	A15	1	0	1	1
	A17	1	0	1	1
	A18	2	1	2	1
		4	0	4	3
	A24	2	0	2	2
Coxsackie	B1	2	0	2	0
	B3	1	1	1	0
Adeno		24	2	24	1
Untyped		8	1	7	5
Total		101	46	87	50

[a] One specimen yielded a poliovirus type 3 and an echo 1 virus.

The adenoviruses were isolated from 11 Houston and 13 Mexican specimens. A " limited " identification of the isolates from Mexico City showed three of them to be of type 4 and one of type 7.

Two of the eight untyped isolates grew only in HK

TABLE 4

COMPARATIVE VIRAL SUSCEPTIBILITY OF THE ATTLESON CELL LINE AND OTHER CELL CULTURES

Virus		Primary isolation				Passage of HK isolates		
		Human kidney	Monkey kidney	WI-38	Attleson	Monkey kidney	WI-38	Attleson
Polio	1	+	+	+	–	+	+	+
	2	+	+	+	–	+	+	+
	3	+	+	–	–	+	+	+
Coxsackie	A9	+	+	+	–	+	+	+
	A18	+	+	–	–	+	+	–
	A20	+	–	–	–	–	+	–
	A24	+	–	+	–	–	+	–
Coxsackie	B3	+	+	–	+	+	+	+
Echo	6	+	+	–	+	+	+	+
	6	+	–	–	–	+	+	+
	8	+	+	+	+	+	+	+
	12	+	–	–	+	+	+	+
	21	+	–	–	–	+	+	–
Adeno		+	–	–	–	+	+	–
		+	–	–	–	+	+	–
		+	–	–	–	+	+	+
		+	–	–	–	+	–	+
		+	–	–	–	+	–	–
		+	–	–	–	–	+	–
Untyped		+	–	–	–	–	+	–
Total		20	8	5	4	16	18	11

and were from Mexican children. The six Houston untyped isolates consisted of one that grew in WI-38 only, four in both HK and WI-38, and one in MK and HK cells. These untyped isolates failed to type with the known enterovirus antisera and may represent new enteroviruses.

Susceptibility of the Attleson cell to enteric viruses

The susceptibility of this human cell strain to enteric viruses was tested in parallel with MK and WI-38 cells by inoculation of rectal swab suspensions from the Mexican series from which virus had previously been isolated in HK cells. The sensitivities of the three cell lines were compared using the first-passage viral material obtained from HK cells. Table 4 gives the results.

The Attleson cell, for primary isolation, was able to produce only four positives—namely, three echoviruses and one group B coxsackievirus. No poliovirus, group A coxsackievirus or adenovirus was recovered. There were eight isolations in MK and five in WI-38 cells and these included a greater range of viral types than was recovered with the Attleson cell. With the HK isolates, the Attleson cells showed better results but these were still not as good as those with WI-38 or MK cells. Furthermore, Attleson cells seemed to be particularly poor for group A coxsackieviruses and adenoviruses.

The WI-38 cells, while inadequate for primary isolation, were specially productive for the adapted virus isolates, allowing 18 of the 20 viruses tested to grow. They may, therefore, be used for virus passage and typing in place of HK cells without great loss of efficiency.

In this small series monkey kidney cell cultures were actually less sensitive for primary isolations, although for passage of established viruses they were largely satisfactory.

DISCUSSION

Different types of cells have been used for the primary isolation of viruses from the enteric tract. For the recovery of the maximum number of viruses it is often necessary to inoculate each specimen into several different cell cultures because no single cell culture is available with susceptibility to all the enteric viruses. The human kidney cell culture, however, comes closest to this ideal.

In this comparative study the human kidney has been shown to be superior to the monkey kidney, which is generally accepted as the cell culture for yielding the greatest variety of viruses from the enteric tract. Of the 100 positive isolations made from 387 rectal swab specimens, 86% were obtained in human kidney cultures. This was almost twice the number obtained from monkey kidney and more than one-and-a-half times that obtained from WI-38 cells. The human kidney surpassed the monkey kidney and WI-38 cells not only in the number of positive isolations but also in the range of viruses isolated. In contrast to the other two cell lines its susceptibility to infection by the group A coxsackie-viruses and adenoviruses was clearly demonstrated. It is highly susceptible to the echoviruses, as shown here and by others (Hsiung, 1962). Lately it has been used successfully for the propagation of rhinoviruses (Tyrrell & Parsons, 1960; Hamre & Procknow, 1961).

Human kidney cultures are easy to prepare and to maintain as stationary or roller cultures. The kidneys from foetuses, stillbirths and young infants have given goods yields of cells, and cultures from all three sources were equally satisfactory for virus growth. Secondary cultures of human kidney cells have also been used with no detectable loss of susceptibility.

The availability of a regular supply of human kidneys could be a problem in some countries and this appears to be the only factor militating against having them as the main tissue culture system in the laboratory. A supply of human kidneys, even on a limited scale, is considered worth obtaining, for their use could then be reserved for primary isolations while other follow-up investigations could be carried out in an alternative cell culture. For this latter purpose, the WI-38 cells may be recommended. They are readily available and can be maintained with little difficulty in the laboratory. While they are not so sensitive as human kidney cells for primary isolation, they do support the growth of a variety of enteric viruses after these have been initially established in human kidney. This combination of human kidney and WI-38 cells should fulfil adequately the tissue culture requirements for for most studies on the enteric viruses.

The Attleson cell line did not compare favourably with the monkey kidney or the human diploid cells and is not recommended for propagation of enteric viruses.

RÉSUMÉ

Les avantages respectifs de différentes cultures cellulaires pour l'isolement des virus entériques font l'objet de cette étude comparative.

Sur un total de 387 prélèvements rectaux, 100 isolements positifs ont été obtenus, montrant la présence des virus poliomyélitiques des types 1, 2 et 3, de 12 variétés d'échovirus, de sept types de coxsackievirus A, de deux types de coxsackievirus B, de 24 souches d'adénovirus et de 8 autres virus non identifiés.

Les cultures de tissu rénal humain ont démontré leur supériorité en donnant un pourcentage d'isolements positifs de 86, alors que pour les cultures de tissu rénal de singe et les cultures de tissu pulmonaire humain (souche WI-38), les pourcentages n'atteignaient respectivement que 46 et 50. Elles ont permis d'autre part d'isoler une gamme plus étendue de virus et, à l'opposé des deux autres lignées cellulaires, elles se sont montrées très favorables à l'isolement des coxsackievirus A et des adénovirus, et très sensibles aux échovirus.

Les auteurs signalent la facilité de la préparation de ces cultures à partir de reins de fœtus, d'enfants mort-nés ou de jeunes enfants, et de leur maintien sous forme de cultures stationnaires en tubes ou de cultures en tubes roulants. Lors de l'emploi de cultures secon-

daires de cellules rénales humaines, on n'a pas constaté de perte appréciable de sensibilité à l'infection par les virus.

Les auteurs suggèrent qu'en cas de difficultés d'approvisionnement, les cultures de cellules rénales humaines soient réservées aux isolements primaires de virus, les recherches ultérieures étant effectuées sur cultures de cellules WI-38 qui permettent le développement de bon nombre de virus entériques après premier passage sur rein humain.

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