Cultivation of Enteroviruses in Hamster Kidney Tissue Culture. (24612)

ALMEN L. BARRON AND DAVID T. KARZON*
Depts. of Bacteriology and Immunology, and Pediatrics, University of Buffalo School of Medicine, and Statler Research Labs., Children's Hospital, Buffalo, N. Y.

Enteroviruses(1) have recently been defined as consisting of polioviruses, Coxsackie and ECHO (enteric cytopathogenic human orphan) groups. ECHO viruses, with few exceptions, do not produce overt disease in monkeys, suckling mice, or other laboratory animals. To date, only tissue cultures of human or simian sources have proved of practical value in growth of enteroviruses. Interest in extending the range of infection of this group in tissue culture led to present investigation of the use of hamster kidney tissue culture. Hamster tissue was selected because of availability of the hamster, and the known susceptibility of suckling hamsters to Coxsackie infection. A cytopathogenic effect was obtained with certain enteroviruses and other viruses in this tissue culture system.

Materials and methods. Preparation of tissue cultures. Kidneys from adult golden Syrian hamsters were minced, trypsinized, and suspended in essentially the same manner as for preparation of monkey kidney tissue cultures(2). The cells were resuspended in growth medium consisting of 0.5% lactalbumin hydrolysate, 2% calf serum and 97.5% Hanks' balanced salt solution and 200 units penicillin, 200 μg streptomycin and 100 units mycostatin/ml. Prior to dispensing the cells, calf serum was added to make final concentration 8%. Following this method, the cells resuspended more evenly and had less tendency to aggregate than if they were resuspended in 8% calf serum immediately. Tube cultures were prepared with 1 ml of a suspension containing 500,000 cells. After 4 days' incubation at 36°C, the cultures were exchanged with above medium containing 4% calf serum.

At time of virus inoculation, the growth medium was replaced with 0.9 ml maintenance medium, consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract (Difco), 0.2% bovine albumin (Fraction V, Armour & Co.), 2% calf serum, 97.2% Earle's saline, with antibiotics added as above. Cultures were inoculated with 0.1 ml undiluted stock virus. They were observed daily for 7 days. All viruses† were passaged 3 times in hamster kidney tissue culture before results were considered negative for virus cytopathogenic effect (CPE).

Results. Preparation of hamster kidney tissue cultures. Cultures were prepared using 4-8 kidneys. The yield of cells varied in different experiments, from 1.7-3.8 x 10^7 cells/kidney. The monolayer which developed after 6-7 days was not coalescent. Examination of stained preparations revealed the presence of 2 morphologically different cells (Fig. 1). Cultures consisted of spindle-shaped cells and small groups of large polygonal-shaped cells, which had a syncytial-like appearance. Nuclei of large polygonal-shaped cells were oval containing 1 to 10 elongated and irregularly shaped nucleoli. During 7 day observation period following inoculation, there was gradual degeneration of the culture, particularly after 4th-5th day. Increasing serum concen-

* Markle Scholar in Medical Science.

† ECHO virus prototypes were obtained through courtesy of Committee on Enteroviruses(1). Poliovirus types I (Mahoney), II (MEF-1), III (Saukett) were obtained from Connaught Medical Research Labs, Toronto. Coxsackie prototypes A9, B1-B5 were obtained from N. Y. State Labs, Albany. ECHO 9 isolates through courtesy of Dr. C. E. van Rooyen, Halifax, (Linda S., Donald S.); Dr. O. Lahelle, Norway (V4162-7); Dr. A. J. Rhodes, Toronto, Canadian strains (309-56, 286-56), English strains (Squirrel, Haynes); Dr. R. Wigand, Cincinnati (Flack, Daniels). ECHO 9 (Hill) strain from Dr. W. McD. Hammon, Pittsburgh; Dr. S. S. Kalter, Montgomery; Dr. J. L. Melnick, Houston; Dr. H. A. Wenner, Kansas City. Other viruses studied were maintained in chick embryos in this laboratory: herpes simplex, HF strain; vaccinia, commercial calf-lymph vaccine; Newcastle disease virus-B strain; canine distemper virus—Onderstepoort strain; influenza A, Jap/305/57 and PR8/34.

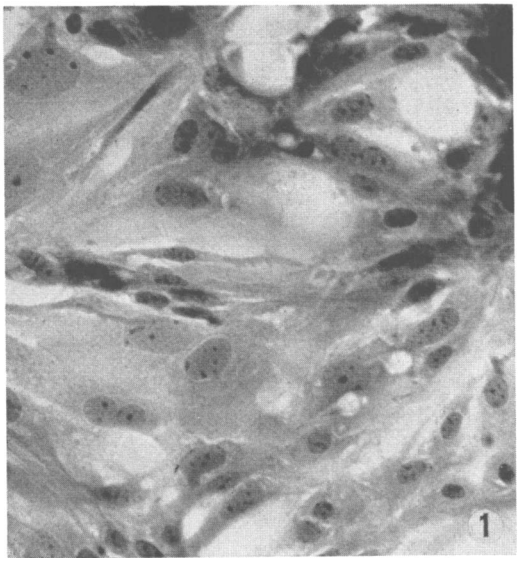

FIG. 1. Coverslip culture of hamster kidney, fixed in Zenker's solution and stained with hematoxylin-eosin. Large polygonal-shaped cells are surrounded by spindle-shaped cells. ×500.

tration to 3% aided maintenance of hamster cells. Eagle's medium in a base of Hanks' balanced salt solution or Earle's saline, plus 2%-3% calf serum also proved a satisfactory maintenance medium.

Susceptibility of hamster kidney cultures to enteroviruses. The results obtained with the enterovirus prototypes are summarized in Table I. None of the 3 poliovirus types produced any CPE. Of the 19 ECHO prototypes, a positive CPE was obtained for ECHO 10 and ECHO 9. Coxsackie viruses B1-B4 produced a rapid CPE (Fig. 2, 3), whereas B5 was slow in manifesting a cytopathogenic response. Strains other than prototypes of Coxsackie and ECHO viruses isolated in monkey kidney in this laboratory were inoculated into hamster cultures. Four Buffalo isolates of Coxsackie B2, 1 of B3, 8 of B4 and 13 of B5 were tested. All strains tested were positive. A 1957 Coxsackie B5 strain behaved like the prototype, producing a very slow degeneration of the cells. However, B5 strains isolated in 1958 in monkey kidney, produced a rapid CPE in hamster. The 2 A9 strains tested were negative, whereas the prototype had been adapted to hamster culture. Although the prototype ECHO 9 proved positive, most ECHO 9 strains were not cytopathogenic. Twenty-seven strains other than the prototype were tested, including 6 isolates from Norway, 2 from Halifax, Canada, 2 from Toronto, Canada, 2 from England, 2 from Ohio, and 13 isolates from Buffalo. Only 2 of these strains were positive. One was from Ohio (strain Daniels), and had been isolated contemporaneously with the prototype Hill strain (3). The other was isolated in Buffalo in 1957 from a household associate of a case of aseptic meningitis. In addition, prototype ECHO 9 (Hill) strain, obtained from 4 other laboratories and passaged once in this laboratory in monkey kidney, was cytopathogenic in hamster kidney tissue cultures. After 3 negative passages in hamster kidney, selected viruses were tested for 'silent growth', *i.e.* virus multiplication without manifestation of cytopathogenic effect, by inoculation of tissue culture fluid into monkey kidney tissue cultures. Negative results were obtained with ECHO 6 (D'Amori) and 10 non-cytopathogenic ECHO 9 strains. Polio virus type II was recoverable

TABLE I. Susceptibility of Hamster Kidney Tissue Cultures to Prototype Enteroviruses and Other Viruses.

Virus	Hamster passage 1	2	3
Enteroviruses			
Poliovirus types I, II, III	0	0	0
Coxsackie B1-B4	R	R	
B5	S	S	
A9	0	S	M
ECHO types 1-19	0	0	0
except: type 9	S	M	R
" 10	M	R	
Other viruses			
Herpes simplex	R		
Influenza PR8	0	0	0
Asian	S	M	R
Canine distemper	0	0	0
Vaccinia	R		
Newcastle disease virus	R	R	R

Viral cytopathogenic effect:

M—*moderate,* CPE not definite until 48-72 hr after inoculation; monolayer destroyed, but may take 96 hr after infection.

R—*rapid,* CPE definite at 24-48 hr after inoculation; monolayer destroyed within 48 hr after infection.

S—*slow,* CPE definite at 72 hr after inoculation; monolayer not destroyed 72 hr after infection.

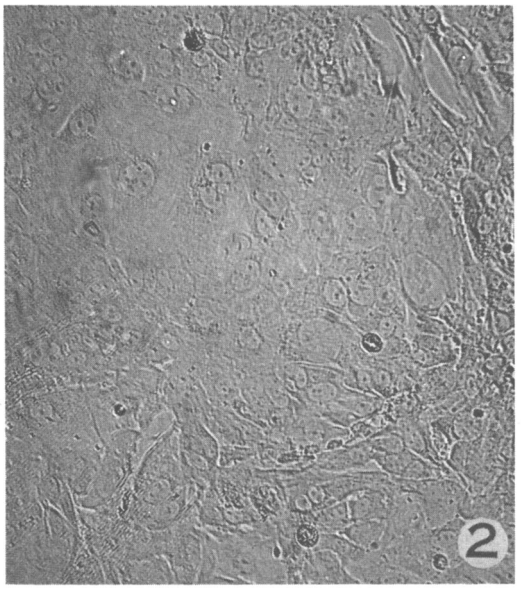

FIG. 2. Unstained T flask culture of hamster kidney. ×100.

in monkey kidney after 3 negative hamster kidney passages but not after 5. Polioviruses types I and III, after 3 hamster passages, were negative in monkey kidney. Negative results for canine distemper and influenza PR8 were confirmed by chick embryo infectivity tests.

Comparative sensitivity of hamster and monkey kidney tissue cultures. ECHO 9 (Hill), Coxsackie B2 and ECHO 10 stock monkey kidney viruses and hamster passaged viruses were titrated simultaneously in hamster and monkey kidney tissue cultures (Table II). ECHO 9 monkey kidney virus which originally titered 6 log TCD_{50} higher in monkey kidney became equally infective for both systems following 5 hamster kidney passages.

TABLE II. Comparative Sensitivity of Hamster and Monkey Kidney Tissue Cultures.

Virus	Tissue culture passage No.		Titer*	
	Monkey	Hamster	Monkey	Hamster
ECHO 9 (Hill)	13	0	6.8	.8
	13	5	4.3	4.3
Coxsackie B2	6	0	5.8	4.8
	6	2	6.5	6.8
ECHO 10	16	0	3.5	4.5
	16	3	3.5	3.8

* Log TCD_{50}/.1 ml.

Coxsackie B2 and ECHO 10 monkey kidney viruses were equally infective for both systems and this was unaltered after hamster kidney passage. Preliminary experiments, not presented here, have shown that monkey kidney and hamster kidney trypsinized cell suspensions can be mixed and grown together to form a monolayer. The large polygonal hamster cells were easily recognized in mixed cultures. Selective virus infection of monkey kidney cells in the mixed population has been obtained with poliovirus. Complete destruction of monkey kidney cells was noted, leaving hamster cells apparently unaffected.

Susceptibility of hamster kidney cultures for isolation of viruses from clinical specimens. Isolation attempts were made simultaneously in hamster and monkey kidney tissue cultures, using clinical specimens from which Coxsackie viruses (B2, B3, B4, B5) had been isolated in monkey kidney tissue cultures during routine diagnostic studies in 1956-57. Stool suspensions, rectal and throat swabs and cerebrospinal fluid specimens were prepared as previously reported(4). Seven of 8 specimens tested were positive in monkey kidney whereas 4 were positive in hamster. During a study of aseptic meningitis in western New York in 1957, hamster kidney tissue

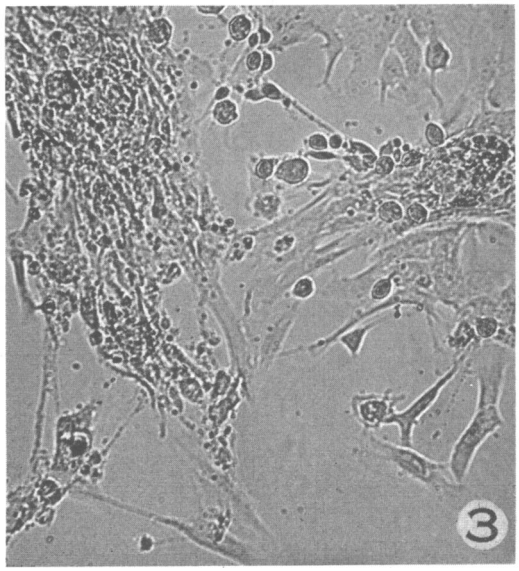

FIG. 3. Unstained T flask culture infected with Coxsackie B1 showing typical CPE. ×100.

cultures were used in diagnostic screening of monkey kidney isolates. Some isolates were cytopathogenic in hamster kidney and others were not, which suggested that the outbreak was due to a mixed group of enteroviruses, probably including Coxsackie viruses and ECHO viruses. Serologic tests later verified that hamster cytopathogenic isolates were, in all cases, Coxsackie viruses of group B, mainly type 4. Those isolates negative in hamster were identified as ECHO viruses, mainly ECHO type 9.

Discussion. Use of hamster kidney tissue culture in the study of several viruses has recently been described. Kissling(5) and Diercks and Hammon(6) have reported on growth of arthropod-borne viruses in this tissue culture system. The present report supplements the list of viruses cytopathogenic in hamster kidney tissue cultures, particularly in reference to members of the enterovirus group. Coxsackie viruses and ECHO viruses types 9 and 10 were cytopathogenic in hamster cultures. Polioviruses did not multiply, which is in agreement with results of Diercks and Hammon(6).

Wright(7) obtained a cytopathogenic effect in hamster kidney cultures inoculated with fluid of monkey kidney cultures containing measles virus isolated from a patient. Guerin and Guerin(8) studied susceptibility of adult porcine kidney tissue cultures to enteroviruses. The results are in agreement with those obtained for hamster kidney with the exceptions of Coxsackie A9 and ECHO 9, which were negative in porcine tissue. Warren and Cutchins(9) tested polioviruses and Coxsackie viruses in bovine embryonic tissue culture with negative results. The ECHO viruses were not examined in their study. Sheffield and Churcher(10) reported serial propagation of poliovirus in cells derived from rabbit embryo kidney. The interrelationships of members of enterovirus group to human and animal infection might be further explored with above tissue culture systems, since a gradient of sensitivity to infection is suggested from the results.

The results presented here and reported by others, show that hamster kidney tissue cultures can serve a very useful function in the diagnostic virology laboratory. This is demonstrated by the wide range of susceptibility of this system to virus infection and availability of tissue to most laboratories. In this study, recovery of Coxsackie virus in hamster kidney culture from clinical specimens was not as efficient as in monkey kidney.

Variation in susceptibility of hamster kidney tissue culture to infection with ECHO 9 isolates is difficult to explain. Other than the prototype Hill strain, only 2 strains of 27 examined were positive. Since approximately 10^6 monkey kidney TCD_{50} doses were required to initiate infection in hamster kidney with ECHO 9 (Hill) strain, susceptibility to infection seems to be of low order. This is supported by the fact that 10 ECHO 9 strains which failed to produce a cytopathogenic effect in hamster kidney did not grow 'silently' as shown by tests in monkey kidney. Certain ECHO 9 strains infect suckling mice, causing myositis typical of Group A Coxsackie infection, and it has been reported that large doses of virus are necessary to initiate infection in this host(11). The adaptability of a few ECHO 9 isolates to hamster kidney tissue culture further suggests the possibility of relationship of ECHO 9 to the Coxsackie group. The positive results with ECHO 10 supplement the evidence that this virus is biologically distinct from other members of the ECHO group. ECHO 10 virus has been shown to infect suckling mice and chick embryos and to be larger in size than other ECHO viruses (12,13). In addition this virus produces a characteristic pathologic change in monkey kidney tissue culture which differentiates it from the other enteroviruses(14).

Summary. Susceptibility of hamster kidney tissue culture to enteroviruses was studied. A cytopathogenic effect was obtained with the prototype Coxsackie viruses (A9, B1-B5) and prototype ECHO virus types 9 and 10. Variability of infectivity was shown for ECHO 9 isolates, only 2 of 27 positive. Other ECHO serotypes and polioviruses were negative. Strains of herpes simplex, vaccinia, Newcastle disease virus and influenza (Asian), also produced a cytopathogenic effect in hamster kid-

neys. Use of hamster kidney tissue cultures as a selective system in identification of agents from cases of aseptic meningitis was reported.

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Incidence of Liver Tumors in Male and Female Rats Fed Carcinogenic Azo Dyes. (24613)

A. S. MULAY AND R. W. O'GARA

Dept. Health, Education and Welfare, P.H.S., N.I.H., Nat. Cancer Inst., Lab. of Pathology, Bethesda, Md.

Sex and sex hormones have an influence on carcinogenesis in the liver. Female mice develop more liver tumors than males following administration of *O*-aminoazotoluene(1). Testosterone administration lowers this incidence (2), and stilbesterol-treated female mice develop liver tumors more readily than untreated females when fed diets containing *O*-aminoazotoluene(3). However, male rats of several strains are more susceptible to development of liver tumors than females, when they are fed diets containing acetylaminofluorene (4,5,6). Working with carcinogenic azo dyes. Rumsfeld *et al.*(7) find a higher incidence of liver tumors in male than in female rats fed 3'-methyl-4-dimethylaminoazobenzene or 4'-fluoro-4-dimethylaminoazobenzene. The present report describes the relative incidence of liver tumors in male and female Osborne-Mendel rats fed azo dyes, *p*-dimethylaminobenzene-1-azo-1-naphthalene (DAN), *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N), or *p*-dimethylaminoazobenzene (DAB).

Materials and methods. Male and female Osborne-Mendel rats, 3-4 months old, were fed a semisynthetic diet containing *p*-dimethylaminobenzene - 1 - azo - 1 - naphthalene (DAN), *p* - dimethylaminobenzene - 1-azo-2-naphthalene (DA-2-N), or *p*-dimethylaminoazobenzene (DAB). The diet consisted of cerulose sugar 78%, vitamin-low casein 12%, salt mixture 4%, corn oil 5%, and vitamin mixture 1%. The composition of the vitamin mixture in mg % was thiamine hydrochloride 30, riboflavin 20, choline chloride 300, calcium pantothenate 70, pyridoxine hydrochloride 25, and nicotinic acid 50. In addition each animal received one drop of halibut liver oil once a week. Treatment of rats in each experiment is tabulated in Table I. *DAN*: Seventy-five male and 60 female rats received 0.075% DAN in the diet for 300 days. Ten rats each received respectively, 0.15, 0.3, and 0.6% DAN for 300 days. *DA-2-N*: Forty male and 24 female rats were fed a diet containing 0.075% DA-2-N for 230 days. *DAB*: Eighty male and 40 female rats were fed diets containing 0.06% DAB. Another group of