Viruses in Cell Cultures of Kidneys of Children with Congenital Heart Malformations and Other Diseases.* (29607)

MATILDA BENYESH-MELNICK, HARVEY S. ROSENBERG AND BERNICE WATSON
(Introduced by J. L. Melnick)
Departments of Virology and Epidemiology, Pathology, and Pediatrics, Baylor University College of Medicine and Texas Children's Hospital, Houston

Occult viruses have been recovered from man or monkeys by means of cultivation *in vitro* of cells from various organs(1-8). In man the virus-yielding organs have thus far been mainly tonsils and adenoids(1,2,4) or lymph nodes(3), while in the monkey the kidneys have been shown to be a main reservoir of latent viruses(5-8). The influence of the agents isolated from the kidney on the carrier monkey is not as yet understood. Nevertheless, the findings in the monkey served as a model for the systematic search for hidden viruses in man.

The present communication summarizes results of viral isolations from cell cultures derived from kidneys, and occasionally other organs, obtained at autopsy from children several days to 15 years of age, during a period of 2 years (October 1961-September 1963). The detailed clinical and pathological analysis of the material studied is a subject of a separate publication.

Materials and methods. Procurement of autopsy material. Regardless of the clinical diagnosis at time of autopsy, one intact kidney was placed in a sterile jar containing Melnick's lactalbumin hydrolysate medium, with 5-fold the usual concentration of antibiotics (penicillin 500 U/ml, streptomycin 500 μg/ml). Occasionally portions of other organs were also obtained; each organ was placed in a separate jar, and held at 4°C. Most of the kidneys were received and processed in the tissue culture laboratory within 2-3 hours after autopsies but some were processed 24 to 48 hours later.

Kidney cell cultures. One portion of each kidney was minced and stored at —20°C and the rest was trypsinized by the usual methods employed in this laboratory for trypsinization of primary monkey kidney(9). Cultures were seeded at a concentration of 5×10^5 viable cells per ml into: tubes (1 ml/tube) for continuous observations; 16 oz bottles (40 ml/bottle) for preparation of secondary cultures, and 60 mm plastic (Falcon) petri dishes (5 ml/dish) containing 5 or 6 round coverslips for histological examination. The petri dishes were incubated in a 5% CO_2 atmosphere.

The tube and bottle cultures were started in Melnick's medium A with 20% bovine fetal serum and a low sodium bicarbonate concentration (0.375 g/liter), for this medium was found to be superior to Eagle's medium for initiation of growth. An intermediate growth medium, consisting of Eagle's medium with 10% heat-inactivated calf serum and a bicarbonate concentration of 0.75 g per liter, was utilized after initiation of monolayer formation (usually 3-5 days after seeding) and until complete monolayers were formed. The same medium was used for propagation of secondary and tertiary cultures. Tube cultures (primary, secondary, and tertiary) kept for prolonged observation were maintained in either Eagle's medium, or Melnick's medium B, both with 5% heat-inactivated calf serum and bicarbonate at a concentration of 3 g per liter.

Primary, secondary, and tertiary petri dish cultures were grown in Eagle's medium with 20% bovine fetal serum, 3.45 g bicarbonate per liter and maintained in the same medium, but with 5 or 10% heat-inactivated calf serum.

All fluids contained 100 units of penicillin and 100 μg streptomycin per milliliter.

Routinely, all the kidneys were carried and maintained as primary and secondary cultures. In the initial stages of the study, tertiary cultures were prepared from all secondary cultures, but thereafter they were made

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TABLE I. Success of Kidney Cell Propagation as Related to Patient's Age and Time Interval Between Autopsy and Trypsinization.

	Interval between autopsy and trypsinization							
	1–6 hr		7–24 hr		25–48 hr		Totals	
Patient's age	No. pos*/ No. tested	%	No. pos/ No. tested	%	No. pos/ No. tested	%	No. pos/ No. tested	%
0– 6 mo	44/56	78	6/10	60	7/11	64	57/77	74
7–12 "	7/15	47	2/5	40	—	—	9/20	45
1– 5 yr	12/33	37	2/6	33	0/3	0	14/42	33
6–15 "	4/12	33	0/5	0	0/2	0	4/19	21
All ages	67/116	58	10/26	39	7/16	44	84/158	53

* Positive = successfully propagated in culture.

only from those questionable for cytopathic changes. Twice a week the medium of all cultures was changed, and they were examined for overt cytopathic changes. They were observed for at least 2 months. Periodically, coverslip preparations were stained with hematoxylin and eosin for more detailed examination.

Identification of cytopathic agents. Human embryonic kidney (HEK) or lung (HEL) cell cultures were used for subculturing of fluids or frozen and thawed cells and supernatant from cultures showing cytopathic changes. Blind passages of cultures, questionable for cytopathic changes, were done also in HEK and/or HEL cells.

The coxsackie virus and measles virus isolates were serologically identified employing neutralization tests with specific antisera in HEK cells. The identification of the cytomegalovirus and varicella virus isolates was made as described(10,11) employing passage material in HEL cells.

Results. During the period Oct. 1961-Sept. 1963, kidneys taken at autopsy from 187 children were received for cell cultivation and viral studies. Of those, 29 (15%) became contaminated within 24 hours after seeding due to heavy infestation of the tissue with bacteria or yeast. These contaminated specimens, most of which came from older children with chronic diseases or children with leukemia, were excluded from the analysis of the study. Of the remaining 158 kidneys processed, 84 (53%) were successfully propagated in culture and studied further.

The percentage of successfully propagated kidney cell cultures decreased with the increase in age of the patient (Table I). While 74% of the kidneys obtained from children under 6 months of age grew, only 21% of the 19 childen over 5 years of age yielded viable cell cultures. The results did not seem to depend upon the time interval between autopsy and trypsinization, especially for kidneys obtained from the very young children. Even though 11 of the kidneys obtained from babies under 6 months of age (Table I) were not processed until 25-48 hours after autopsy was performed, 7 (64%) still grew; this compared favorably to the 44 (78%) successfully propagated kidneys obtained from 56 children of the same group and trypsinized within 1-6 hours after autopsy. Of the kidneys obtained from children 6-15 years of age, however, only those processed early after autopsy were successfully propagated.

In babies under 6 months of age, there was no relationship between the disease causing the death of the child and the success of kidney cell propagation (Table II). In the older children, although the number of children within the individual age groups was not large, there was a suggestion that kidneys of children with congenital malformations grew more readily. Thus, cell cultures were obtained from 50% to 67% of the kidneys taken from children over 7 months of age who had congenital malformations. Only a few of the kidneys obtained from children within the same age range, but with other disease entities, were successfully propagated.

Regardless of the age or diagnosis of the child, once the kidney cells established themselves in culture, they grew progressively and could be maintained as primary, secondary,

TABLE II. Success of Kidney Cell Propagation as Related to Diagnosis and Patient's Age.

	Patient's age									
	0–6 mo		7–12 mo		1–5 yr		6–15 yr		All ages	
Diagnosis	No. pos*/ No. tested	%	No. pos/ No. tested	%	No. pos/ No. tested	%	No. pos/ No. tested	%	No. pos/ No. tested	%
Congenital malformations	35/46	76	6/9	67	9/18	50	2/3	67	52/76	69
Infectious diseases	13/17	77	2/8	25	1/4	25	1/2	50	17/31	55
Leukemias and solid tumors	1/2	50	—	—	1/8	12	1/6	17	3/16	19
Miscellaneous	8/12	67	1/3	33	3/12	25	0/8	0	12/35	34
Totals	57/77	74	9/20	42	14/42	33	4/19	21	84/158	53

* Positive = successfully propagated in culture.

and tertiary cultures for long periods. A common feature for most of the kidney cells propagated was that initially the cultures seemed to be composed of epithelial cells. With time and serial passage, especially in cultures in stoppered vessels, the majority of the cells in the cultures assumed a fibroblastic appearance. No attempts were made to establish permanent cell lines from any of the kidney cell cultures.

In the initial stages of the study, blind passages of supernatant fluids were carried out on human embryonic kidney cells. Usually 3 harvests from each culture were tested: the first fluid collected after the culture was seeded, fluid taken a month later, and frozen and thawed cells and supernatant taken at the end of the observation period, usually 60 days after seeding. Forty primary kidney cultures, 39 secondary cultures prepared from the same 40 primary, and 30 tertiary cultures prepared from the 39 secondary ones, were tested in this fashion. None of the blind passages yielded virus. This approach was therefore abandoned in the further stages of the study.

Occasionally other organs such as thymus, spleen, lung, etc., obtained from the same patients, were propagated in culture in the same fashion as were the kidney cells. The number of different organs processed is not sufficient to warrant a separate summary. However, several organs from different patients yielded cytopathic agents and will be discussed.

Cytopathic agents appeared spontaneously in cultures of kidneys and/or other organs obtained from 9 of the 66 children under one year of age, while none of the cultures from older children were positive. Three of the 9 agents were identified as cytomegalovirus, 3 as adenovirus (types 1, 2 and 7) and one each were measles, varicella, and coxsackie B1 (Tables III, IV).

Table III summarizes some of the observations on the appearance and development of the cytopathic agents in cell cultures of kidneys and other organs, as well as the results of attempts to isolate the same viruses from 20% suspensions prepared at a later date from frozen samples of the same organs and inoculated onto human embryonic kidney (HEK) and/or human embryonic lung (HEL) cells.

The advantage of preparing and observing not only primary, but also secondary and tertiary cell cultures, is evident from the results obtained with the first cytomegalovirus shown (#87). This virus was isolated from the kidney of a one-month-old baby who had expired after surgery performed to repair a congenital heart defect (Table IV). One hundred per cent of the secondary cultures (about 20 were used), prepared from 16 day old primary cultures, exhibited typical cytomegalovirus cytopathic effect 18 days after seeding (34 days after seeding of the primary cultures). On the other hand, only 22% of the primary cultures observed for long periods exhibited the same type of cytopathology, and at a much later date (50 days after seeding). The spread of CPE in the

tertiary cultures was even faster than in the secondary cultures (5 days after seeding). It is obvious that the original kidney cell population contained a very small proportion of infected cells and that serial cell propagation accelerated the spread of infection and the appearance of CPE.

In the case of the adenovirus type 2 isolate (#53), the number of virus-containing cells among the original kidney cells seeded must have been very small for only a few of the primary cultures exhibited cytopathic changes. None of the tubes prepared from the negative 16 oz bottle, or the tertiary cultures prepared from the secondary cultures, revealed the virus. However, the cytopathic agent found in the primary culture was readily passed and identified on HEK cells as

TABLE III. Detection of Cytopathogenic Agents in Cell Cultures and in Suspensions of Organs Obtained from Children at Autopsy.

Virus isolated (child No.)	Organ propagated	Primary cultures		Time ----> in days	Secondary cultures		Time ----> in days	Tertiary cultures		Virus isolation from 20% organ suspension on cultures of:	
		% Pos	Day CPE first observed		% Pos	Day CPE first observed		% Pos	Day CPE first observed	HEK*	HEL†
Cytomegalo:											
(D.P.$_{97}$)	Kidney	22	50	16	100	18	17	100	5	0	0
(B.B.$_{127}$)	"	100	11	12	100	4	5	100	1	0	0
(M.P.$_{168}$)	Kidney	0		30	0			ND		0	0
	Spleen	0			ND			ND		0	0
	Lung	100	19	26	50	10		ND		0	0
Adeno-1: (D.P.$_{140}$)	Kidney	100	8		ND			ND		0	ND
Adeno-2: (H.R.$_{53}$)	"	20	12–20	21	0‡		27	0		0	0
Adeno-7: (C.R.$_{56}$)	"	100	8		ND			ND		+§	ND
Measles: (P.M.$_{138}$)	Kidney	0		19	10	69		ND		0	0
	Spleen	100	81	83	100	2		ND		0	0
Varicella: (T.C.$_{182}$)	Kidney	40	14		ND			ND		0	0
	Lung	0		36	0		55	0		ND	ND
Coxs. B1: (R.O.$_{158}$)	Kidney	100	7		ND			ND		+‖	ND
	Liver	ND			ND			ND		+¶	ND

* HEK = human embryonic kidney.
† HEL = human embryonic lung.
‡ These secondary cultures were prepared from negative primary cultures.
§ Described in detail previously(12).
‖ $10^{4.5}$ TCD_{50} per gram tissue.
¶ $10^{5.9}$ TCD_{50} per gram tissue.

TABLE IV. Clinical and Pathological Findings in 9 Patients Yielding Cytopathic Agents.

Child & No.	Age	Sex	Date	Clinical diagnosis	Viral type inclusion found at autopsy in:	Virological findings	
						Virus type isolated	Organ yielding virus
D.P.$_{87}$	1 mo	♂	8/20/62	Congenital heart disease: 1. Atrial septal defect 2. Congenital mitral stenosis	Kidney Lung	Cytomegalo-	Kidney
B.B.$_{127}$	1 day	♀	1/ 4/63	Omphalocele	Generalized	"	"
M.P.$_{168}$	6 wk	♂	5/ 8/63	1. Congenital heart disease 2. Congestive failure	None	"	Lung
D.P.$_{140}$	13 mo	♂	2/ 4/63	Transposition of the great vessels	"	Adeno-1	Kidney
H.R.$_{53}$	4 mo	♂	4/19/62	1. Coarctation of the aorta 2. Pneumonia	"	Adeno-2	"
C.R.$_{56}$*	5½ mo	♀	5/ 1/62	Pneumonia	Lung Liver	Adeno-7	Kidney, Spleen, Lung, Liver
P.M.$_{136}$	10 mo	♀	1/31/63	1. Measles exposure 2. Pneumonia	Lung	Measles	Kidney Spleen
T.C.$_{182}$	2 wk	♂	6/24/63	1. Prematurity 2. Congenital varicella	Generalized	Varicella	Kidney
R.O.$_{189}$	1 wk	♂	7/22/63	1. Myocarditis 2. Icterus	None	Coxsackie B1	Kidney Liver

* Described in detail previously(12).

adenovirus type 2. The possibility of laboratory cross contamination can be easily ruled out, for this was the first virus isolated in the study. As seen in Table IV, this was the first positive kidney and was received on 4/19/62. Furthermore, no work with adenovirus type 2 was going on in this or any other part of the laboratory.

The same general pattern of isolation was true for the third cytomegalovirus (#168), the measles virus (#136), and the varicella virus (#182). The longest period of latency in culture was observed with the measles virus (81 days after primary seeding of the spleen cells). Cytomegalovirus (#127) was isolated from a one-day-old child who had died with omphalocele and generalized cytomegalic inclusion disease; the cytopathic effect appeared very early (11 days) in all the primary cultures and progressed rapidly (Table III).

The pattern of isolation for adenovirus types 1 and 7 and coxsackie virus B1 was quite different. Cytopathic changes appeared quite early in all of the primary cultures and progressed very rapidly, destroying all the cells in the cultures.

As seen in the last two columns in Table III, only 2 (adeno-7, coxsackie B1) of the 9 viruses recovered by organ propagation were also isolated from 20% organ suspensions. However, no particular effort was made to test organ suspensions routinely. As indicated under *Methods*, portions of the kidneys or other organs were frozen at —20°C. Only if a cytopathic agent became apparent in the propagated cells, did we prepare and test 20% suspensions from the same organ for comparison.

Since some kidneys (*child M.P.*$_{168}$) failed to exhibit virus while other organs (lung) from the same child did, our efforts are now being extended to a systematic study of other organs propagated in the same fashion as the kidneys.

In 5 of the cases studied (B.B.$_{127}$, C.R.$_{56}$, T.C.$_{182}$, and R.O.$_{189}$) there was a definite association between the virus isolated and the disease found at time of demise (Table IV). *Child B.B.*$_{127}$, who expired after surgery to repair a congenital omphalocele was found to have generalized cytomegalic inclusion disease. Cells propagated from its kidney yielded cytomegalovirus. No other

organs were propagated from this patient. *Child C.R.*$_{56}$, described in detail elsewhere (12), expired with pneumonia and disseminated disease due to adenovirus type 7 infection. *Child P.M.*$_{136}$ had been exposed to measles about 2 weeks before expiring with a bilateral giant cell pneumonia. Although no other organs revealed pathological changes at autopsy, measles virus was isolated from the spleen and kidney cell cultures. Unfortunately, the lung cultures had become contaminated 24 hours after seeding and could not be followed. *Child T.C.*$_{182}$, who expired with generalized varicella, was born prematurely while the mother had varicella. The baby had a typical skin rash at birth. The kidney cell cultures yielded varicella virus but the lung cell cultures were negative. *Child R.O.*$_{189}$ was born to a mother who had fever, chills and headache at time of delivery. The child became severely ill at the age of 5 days and died 2 days later with fever, myocarditis, hepatitis, and encephalitis. Coxsackie virus B1 was isolated from the kidney cells grown in culture. Both kidney and liver tissues, tested at a later date, revealed high quantities of the virus, $10^{4.5}$ TCD_{50} per gram and $10^{5.9}$ TCD_{50} per gram, respectively (Table III). Repeated tests of fecal specimens from the mother were negative, but they were taken a month after the child's death and at that time she already had an appreciable antibody titer (1:256) against the coxsackie virus B1 isolated from the baby.

The remaining 4 agents [cytomegaloviruses #87 and #168 and adenoviruses #140 (type 1) and #53 (type 2)] were recovered from young children with congenital heart anomalies. Children D.P.$_{87}$, D.P.$_{140}$, and H.R.$_{53}$, expired after surgery performed to correct the congenital malformations and the fourth child, M.P.$_{168}$, expired with heart failure shortly after admission to the hospital. With the exception of child D.P.$_{87}$, in whom characteristic cytomegalic inclusions were found at autopsy in cells of the kidney and lung, the other 3 children were free of histological evidence of specific viral involvement.

Whether the cytomegalo- and adenoviruses isolated can be implicated as etiological agents of congenital heart defects remains for future study. The 2 cytomegaloviruses (#87 and #168) were indeed isolated from very young infants and it is possible that the infection with the virus was congenital. However, the adenoviruses (#53 and #140) were isolated from older children, 4 and 13 months old, respectively, and might have been accidental passenger viruses harbored by the kidneys of these children with no relationship to their illness at the time of demise.

Discussion. This study was initiated with the aim of uncovering latent cytopathic agents harbored in the kidney of man. There were several reasons for choosing the kidney as a target study organ. As mentioned earlier, primate kidneys have been shown to harbor latent viruses, harmless to the carrier host but capable of causing overt disease when inoculated into a different host. Furthermore, in the past several years, an increasing number of viruses have been found to be carried in the urine of man(13-20). This indicates the possibility that the kidney may be a site of virus multiplication similar to multiplication of SV40 experimentally demonstrated *in vivo* in the green monkey kidney(8). In the latter case, repeated kidney biopsies taken from animals injected with SV40 yielded the virus when grown *in vitro* as cell cultures long after the animals had stopped excreting virus in the urine. Thus, employing the method of cell cultivation to unmask latent viruses present within the cells, we were able to demonstrate cytopathic agents in 9 of the 84 children (11%) yielding kidney cultures or cultures from other organs. In only one case (M.P.$_{168}$), were the kidney cells negative when the lung culture yielded cytomegalovirus. Only in 2 of the cases were we able to isolate the virus (adeno-7 and coxsackie B1) from 20% kidney suspension (Table III); this is in agreement with efforts of others to isolate viruses from suspensions of a variety of human tissues obtained at autopsy, as summarized by Valdes-Dapena and Hummeler(21). However, our efforts were not directed to the comparison between isolations from cells grown in culture and isolations from organ suspensions. It is

possible that the small amounts of virus present originally in the tissue did become inactivated during storage at —20°C until the suspensions were prepared and tested. This period usually ranged from 2-3 months.

Results similar to ours have been reported by Klein and Huang(22) who isolated 5 adenoviruses from over 100 kidney cultures obtained from prematurely born infants. They also failed to isolate the agents from the kidney suspensions. Unfortunately, the autopsy findings in the infants yielding the adenoviruses are not given so that one cannot know whether there were any congenital malformations.

Since our main interest was detection of occult viruses in man, it was disheartening to find that we were not successful in propagation of autopsy material from older children or adults. Because of the larger number of virus exposures which inevitably take place during life, the adult material might have yielded an even richer harvest. We were also unsuccessful in growing material from children expiring with leukemia or other malignancies. Apparently, due to extensive antimetabolite therapy, most of these children expire with very heavy mycotic infections and in our attempts of cell propagation we consistently ended with a pure mold or yeast culture, regardless of the organ under study.

It is noteworthy that McAllister's efforts (4) to unmask hidden viruses in cultures of over 100 surgical biopsy specimens from an array of neoplastic or non-neoplastic tissues taken from a variety of patients failed to demonstrate infectious virus, with the exception of adenoviruses obtained from 6 of 13 tonsils propagated in culture.

Particularly noteworthy in our study is the isolation of 2 cytomegaloviruses and 2 adenoviruses from infants with congenital heart malformations. It is impossible to generalize at this stage and assume that there is a causative relationship between the viruses isolated and the malformations observed. But these findings taken together with the relationship between microcephaly and intrauterine cytomegalovirus infection(23) offer leads for further study.

Summary. A systematic search for occult cytopathic agents in cultures from kidneys and occasionally other organs obtained at autopsy from children several days to 15 years of age, was conducted over a 2-year period. Of the 158 kidneys processed, 84 were successfully propagated in culture. Primary, secondary, and occasionally tertiary cultures were maintained and observed for over 2 months. The percentage of kidneys propagated decreased with increasing age of the patient. Nine of the 66 children less than one year of age yielded spontaneous cytopathic agents in the cell cultures derived from their kidneys and/or other organs. None of the cultures from the 18 older children were positive. Three of the 9 agents were identified as cytomegalovirus, 3 were adenovirus (types 1, 2 and 7) and one each as measles, varicella and coxsackie virus type B1. Only 2 of the 9 isolates (adenovirus type 7 and coxsackie virus type B1) were isolated also from 20% organ suspensions. In 5 of the cases presented, there was a definite association between the virus isolated and the disease of the child. The other 4 agents, 2 cytomegaloviruses and 2 adenoviruses (types 1 and 2) were recovered from infants with congenital heart malformations.

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Effect of Head X-Irradiation on the Uptake of Radiophosphorus by Rat Brains.* (29608)

JOHN R. BERGEN, HEATHER D. SEAY, CHARLES K. LEVY AND WERNER P. KOELLA
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Our primary purpose has been the development of a practical, reliable, quantitative technique for production of increased permeability of the so-called blood-brain barrier (BBB). Such a method would permit not only the study of the physiological effects of increased BBB permeability *per se* but also that of drugs which normally do not penetrate the brain in appreciable amounts. One successful method developed by Purpura *et al* (1) involves freezing of a local area of the brain. The major limitation of this technique is the inability to adapt it to large surface areas and to subcortical sites. A possible practical method circumventing this difficulty utilizes ionizing radiation to produce large scale increases in barrier permeability.

The blood-brain barrier (BBB) now constitutes a functional concept rather than an anatomical entity. It is derived in part from the knowledge that a variety of substances (*e.g.,* metallic cations, phosphates) pass from the blood stream into the brain tissue at very slow rates compared with their entrance into other tissues; in muscle these ions equilibrate in a fraction of the time necessary for equilibration in brain. The mechanisms or structures responsible for this disparity between tissues are unknown and are currently the subject of speculation [reviews by Dobbing (2), Tschirgi(3)].

Numerous investigators have studied the effect of ionizing radiation on uptake and incorporation of a variety of dyes and isotopically labelled compounds(4,5,6,7,8). However, the contradictory results obtained preclude definitive statements as to the effect of radiation on BBB permeability. Explanations for these conflicting reports include: (1) differences in radiation dose; (2) species differences; (3) time of test relative to irradiation; (4) nature of test substance; and (5) differences in treatments of controls.

Radiation has been reported to increase uptake of P^{32} by brain, and to have no effect on uptake. Florsheim and Morton(5) studied the effects of doses up to 20 Krads on *in vitro* and *in vivo* uptake of P^{32} by mouse brain and noted no significant differences between control and experimental animals 24 hours postirradiation. Stajic and co-workers(8), using counting and autoradiographic procedures, confirmed the findings of Florsheim for the 24-hour postirradiation period but found that 20 Krad irradiation to

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