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Titles and authors of the papers are as follows:

a) "The Use of Primary Human Amnion Cell Cultures for the Diagnosis of Poliovirus Infection" - M Jung, J Vesenjak-Hirjan, S Kalcic, D Blatnik, M Matjasic, S Smerdel, E Soos, and B Snoj; Institute of Health, Department of Epidemiology, Virus Laboratory, Ljubljana, Yugoslavia, and School of Public Health "A Stampar", Medical Faculty, Zagreb, Yugoslavia.

b) "Utilization of a New Diploid Cell Strain Derived from Human Embryo Lung Tissue for the Cultivation of Enteroviruses and Measles-Virus" - VI Hozinski, VB Seybil, LB Cypkin, NS Panteleevm, and CM Mazurova; Institute of Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences, USSR, Moscow.

c) "Cultivation of Vaccinia Virus in Human Diploid Cell Strains" J Trlifajova, V Strizova, and F Stastny; Institute of Epidemiology and Microbiology and Institute of Sera and Vaccines, Praha, Czechoslovakia..... UNCLASSIFIED.

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THE USE OF PRIMARY HUMAN AMNION CELL CULTURES FOR THE DIAGNOSIS OF POLIOVIRUS INFECTION

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INTRODUCTION

Multiplication of the three types of poliovirus in primary human amnion (PHA) cell cultures has been reported (Zitcer, Fogh and Dunnebacke, 1955). It has been also demonstrated, that PHA cell cultures were sensitive for cytopathic action of Adenovirus types 1-8, Coxsackie B viruses, herpes simplex, vaccinia, and measles virus (Wilt, Stanfield and Leindl, 1956; Takemoto and Lerner, 1957; Milovanović, Enders and Mirus, 1957). These cultures have been also used for virus isolation attempts. Comparative infectivity titrations in monkey kidney, PHA, and HeLa cell cultures (Lehmann-Grube, 1961) have shown that PHA cell cultures supported growth of ECHO virus types 1, 2, 3, 5-13, 15, 19, 20, 21 and 24, and of types 4, 14, 16, 17, 18, 22 and 23 in a variable degree. It is of considerable interest, that even types 9, 11, 13, 15 and 18 of Coxsackie A group of viruses could be propagated in these cells with satisfactory infectivity titers. Other types of Coxsackie A viruses could be also cultivated after adaptation (Krech, 1961; Lenahan and Wenner, 1961).

The communications, which have been cited here, provide informations on the usefulness of PHA cell cultures for the propagation of many viral agents causing cytopathic effects in tissue culture. There is, however, little evidence on the large scale use of these cells for primary isolation of viruses from clinical specimens. In this communication, we shall present the results of the use of PHA cells for the diagnosis of poliovirus infection. In addition, succesfull primary isolations of viruses other than poliovirus will be reported.

MATERIALS AND METHODS

Pathological specimens were obtained from clinical patients in Slovenia during 1958-1961. Materials were also

collected from patients in Croatia, where poliomyelitis occured in epidemic form in the summer of 1960; during the year 563 cases were reported. In 1961 a vaccination campaigne with Sabin poliovirus vaccine was initiated in Slovenia, while in Croatia the Koprowski type vaccine was employed; in Slovenia the Salk type poliovaccine was also used on a large scale since 1957.

Only a few cases of poliomyelitis-like diseases were reported during 1961-1962 in both countries, but the number of cases with aseptic meningitis remained practically unchanged; a part of these patients was also investigated, and the results included in this report. The participating Virus laboratories were at the National Institute of Health in Ljubljana, Slovenia, and the School of Public Health "A. Štampar" of the Medical Faculty in Zagreb, Croatia. Laboratory methods of testing pathological specimens were similar in both laboratories. In many instances two or more faecal samples were examined in each od the cases under study except during 1960 epidemic, when it was apparent that this would overburden available facilities.

Virus isolations were accomplished in PHA cell cultures, and in HeLa and Detroit-6 cells in some cases. Tissue cultures were prepared according to the methods previously described (Jung, Strah, Blatnik and Vozelj, 1959; Jung, Blatnik, Strauch, Matjašič, Tovornik, Kegu, Drnovšek, Kralj and Zadnik, 1960). Test tube cultures were examined daily for 10-14 days for evidence of cytopathogenicity. Two blind passages were always made with specimens from clinical patients; no blind passages were performed with rectal swabs obtained from healthy children.

The cytopathogenic agents, which were isolated in tissue culture were preliminary grouped by testing the tissue affinity and pathogenicity for suckling mice, as shown in Table 1. Final identification was made in neutralization test with 100 $TCID_{50}$ of the virus, in 0,1 ml, against a limited number of Polio - ECHO - and Coxsackie antisera pools which

were available; they were diluted as to contain at least 20 units of each antibody per 0,1 ml. Neutralization tests were incubated for 3 hours at 37°C, and overnight in the cold-room. The virus-serum mixtures, in 0,2 ml amounts were then inoculated into each of two PHA cell cultures. If neutralization of cytopathogenicity occured, the virus was tested against components of the pool. The poliovirus typing antisera were obtained from Parke, Davis and Co., the Swiss Serum and Vaccine Institute, and from dr. U. Krech, Hilterfingen/Thun. The ECHO antisera types 1, 2, 3, 4, 5, 6, 7, 8, Coxsackie A 9 and 23, and Coxsackie B 1, 2, 3, 4 and 5 were obtained from dr. U. Krech. In 1962, ECHO antisera for types 16 and 18 were also available. Adenoviruses were included into the group by testing the tissue culture supernate for the presence of complement-fixing antigen against a known antiserum. No type identification was made.

For virus titrations serial tenfold dilutions were prepared in the maintenance medium, and 0,1 ml inoculated into each of 2, 3, 4, or 6 PHA cell cultures, according to the experiment. Cell culture tubes for determining the reproductive capacity of polioviruses at different temperatures were kept in specially constructed water baths, maintained at 36°C and 40°C; internal Beckmann type thermometers were used to check the temperature, which varied by ± 0,015°C. Infectivity titers were calculated according to the Kärber method (Kärber, 1931).

Complement-fixation with non-inactivated poliovirus antigens was done with sera from majority of patients; neutralization tests were also performed in many cases.

R E S U L T S

Results of enterovirus isolations in Slovenia, during 1958-1959, from patients with paralytic poliomyelitis and aseptic meningitis in PHA, HeLa and Detroit-6 cell cultures are shown in Table 2. No significant difference was observed in various tissue culture systems, as to poliovirus isolation, although four strains of unidentified enteroviruses could be

detected only in PHA cells. During this period, 39 patients were reported to have paralytic poliomyelitis.

76 cases of paralytic poliomyelitis occured in Slovenia during 1960-1961; materials from 61 patients were tested in the Virus laboratory. Seasonal incidence and vaccination history of these patients are presented in Table 3. Results of poliovirus isolation in various age groups, and of immunologic typing of polioviruses are evident from Tables 4 and 5. In total, clinical diagnosis of paralytic poliomyelitis could be confirmed by virus isolation in 88,5 per cent of cases. It is of considerable interest, that 40,7 per cent of total poliovirus isolates were immunologically related to the type 3; this percentage was even higher in the older children and adults.

During the same period rectal swabs from 1493 healthy children were collected, and tested in PHA cell cultures for the presence of cytopathogenic viruses. Poliovirus strains isolated from this study group, as compared with total number of enterovirus isolates are shown in Table 6. It is evident, that the incidence of poliovirus infection in healthy children was significantly higher in November-December 1960, and February 1961. This is in good egreement with seasonal incidence of paralytic poliomyelitis cases, as presented in Table 3.

Results of virus isolations from healthy children, and clinical patients with paralytic poliomyelitis and aseptic meningitis in Slovenia, during 1960-1961, are evident from Table 7; materials from only a part of aseptic meningitis cases were examined. As shown in the table, 54 strains of polioviruses could be isolated in PHA cell cultures from patients with paralytic poliomyelitis, and 2 strains from aseptic meningitis cases. In contrast, 7 non-poliovirus strains were isolated from non-paralytic patients. These results co-relate well with poliovirus isolations from healthy children, since only types 1 and 3 could be identified by immunologic typing in both study groups, except a single strain of type 2 poliovirus. It is also evident, that 95 strains, cytopathogenic for primary cultures,

were isolated; most of these strains probably belong to the ECHO virus group, since they were non-pathogenic for suckling mice, did not react with Polio and Coxsackie B antisera, and could not be grouped as adenoviruses.

As it was mentionned before, the Republic of Croatia experienced its largest epidemic of poliomyelitis in the history with 563 cases reported during 1960. This epidemic was virologically investigated, and some of the results published elsewhere (Jung, Vesenjak-Hirjan, Lulić, Matjašič, Blatnik, Spalatin and Fryda-Kaurimsky, 1961). In the first group, materials from 64 patients were tested serologically, and in tissue culture. Results of virus isolations in PHA cells are presented in Table 8. Cytopathogenic viruses were isolated from faecal material or CNS tissue in 47 out of 60 patients with paralytic forms of the disease, and from 3 out of 4 non-paralytic patients. The type 1 poliovirus was found in 36 cases. The sensitivity of PHA cell cultures, as evaluated by the time of appearance of cytopathogenic effect, is evident from Table 9; 93 per cent of polioviruses were detected within the first 10 days of incubation at 37°C.

Stools from a second group of 218 patients were tested some months later in PHA and Detroit-6 cell cultures, and 122 strains of cytopathic agents were isolated of which 62 strains in PHA cells, out of 76 specimens, and 60 strains in Detroit-6 cultures, out of 142 specimens. Immunologic typing with poliovirus antisera showed 58 strains to be type 1, 3 type 2, and 8 strains to be type 3 poliovirus.

Comparative infectivity titrations of poliovirus strains in various tissue culture systems have also been performed. Table 10 presents infectivity titers of some standard attenuated and virulent strains of polioviruses in PHA and monkey kidney cells, incubated at 36°C and 40°C, and in HeLa cells, incubated at 36°C. Comparable results were obtained with PHA and monkey kidney cells, although results with HeLa cells were less satisfactory. Tables 11 and 12 show infectivity titers

of type 1 poliovirus strains in various tissue culture systems incubated at 36°C and 40°C. It is evident, that PHA and monkey kidney cells gave comparable results. In HeLa cells the infectivity titers were less satisfactoty, and in some cases they were completely resistant to cytopathic effects of polioviruses. A small number of type 3 polioviruses were also tested, and the results are given in Table 13. The reproductive capacity of type 3 strains was not significantly reduced at 40°C. It must be noted, however, that most of the strains were isolated very late after the poliovaccine was fed.

The effect of temperature 40°C was examined in some cases, by additional incubation at 36°C for 72 hours. As evident from table 14, with majority of strains the infectivity titers were identical or the difference was minimal, not exceeding 1,0 log.

As shown previously, cytopathic agents, other than poliovirus, could be isolated in PHA cell cultures. Infectivity titers of some ECHO viruses are presented in Table 15. Satisfactory titers were obtained with virus types 1(8), 6, 9, and 16. In Table 16 the results of primary non-polio enterovirus isolations, from patients with aseptic meningitis in Croatia and Slovenia, during 1962, have been summarized. In total, 52 strains were isolated, of which 6 ECHO viruses out of 26 strains which were identified. It is of considerable importance, that no polioviruses were found in both countries during 1962.

DISCUSSION

Freshly explanted human amnion cultures consist of normal cells with a basic diploid DNA content, and with a relatively slow mitotic process (Leuchtenberger, Boyer and Strain, 1959). The abundance and availability of placental membranes enables a large scale production of these cultures. On the basis of trypsinization of over 600 membranes, it can be stated, that sucessfully grown cultures are free of spontaneously occuring cytopathic viruses; this fact is of obvious importance when cell cultures are used for primary virus isolations. The PHA

cells can be easily maintained for several weeks with relatively infrequent changes of the nutritient medium. The cytopathic effects of viruses can be easily observed under low power magnification of the microscope. Details of cytopathology in PHA cells have been reported elsewhere (Jung, Strauch, Blatnik, Kresnik, Tovornik, Železnik and Matjašič, 1960; Kresnik and Jung, 1961).

As a results of investigation of 197 patients with paralytic poliomyelitis in Slovenia and Croatia, it can be concluded that PHA cell cultures represent a reliable tool for the diagnosis of poliovirus infection. In addition, Simultaneous virus isolation attempts were made in PHA, HeLa, and Detroit-6 cells. In Slovenia, during 1960-1961, the clinical diagnosis was confirmed by poliovirus isolation in 88,5 per cent of cases under study, ranging from 76,9 per cent in adults, up to 95,0 per cent in children aged 0 - 4. During the 1960 epidemic in Croatia, viruses were isolated in PHA cells in 47 out of 60,and in 62 out of 76 materials, from paralytic poliomyelitis patients, thgt is in 78 and 81 per cent, respectively. These results have been in accordance with previous experience. Virological studies of epidemic poliomyelitis, during 1956, in Chicago and Cook County were made with materials from 664 patients, which were tested for virus isolation in monkey kidney cell cultures; in total, viruses were found in 71 per cent of cases, of which 65 per cent were polioviruses (Nathanson, Thrupp, Hall, Langmuir, Cornell, Forester, Church, Hall, Hildebrand, Shaughnessy and Morrissey, 1959). Enterovirus isolations, including Polio, ECHO, and Coxsackie viruses, which were performed in the same tissue culture system during 1958 epidemic in Detroit, were positive in 73 per cent of paralytic poliomyelitis patients (Brown, Lenz and Agate, 1960). Virus isolations in monkey kidney cell cultures during 1959 poliomyelitis epidemic in Des Moines and Polk County, Iowa, gave positive results for polioviruses in 56 out of 67 paralytic patients (83 per cent) from which stools were available for laboratory investigation; all the isolates belonged to the type 1 poliovirus. The recovery of enteroviruses

from stools of non-paralytic patients was 34,4 per cent; in this group Coxsackie B 2, ECHO 7, and unidentified cytopathogenic agents were also found (Chin, Marine, Hall, Gravelle and Speers, 1961).

These results co-relate well with our experience, although PHA cell cultures were used through the study.

Comparative infectivity titrations with virulent and attenuated poliovirus strains were performed in PHA, monkey kidney, and HeLa cells. PHA and monkey kidney cell systems were equally susceptible to the virus strains under study, while HeLa cells gave results, which were less satisfactory. PHA cells could also be sucessfully used for performing infectivity titrations in cultures incubated at 40°C.

PHA cells were used for primary isolations of polioviruses (Lahelle, 1957; Chadwick, Welsh and Lennette, 1959), ECHO viruses type 6 and 9 (Lahelle, 1957a; Krech and Wulff, 1957; McLean and Melnick, 1957), adenoviruses (Takemoto and Lerner, 1957), and measles virus (Ruckle and Rogers, 1957). In our experience, the following viruses were isolated from patients with paralytic poliomyelitis and aseptic meningitis : poliovirus types 1-3, Coxsackie B virus types 1, 3, and 5, Coxsackie A 9 and 23 (ECHO 9), and some unidentified group A strains, and ECHO virus types 1(8), 2, 3, 6, 7, 9 (Coxsackie A23), and 16. It is also very likely, that many of the viruses, which were not typed and/or identified, belong to ECHO viruses, since only a limited number of ECHO antisera types were available to us. This is especially the case with 95 strains of cytopathogenic agents, presented in table 7, which were isolated from healthy children, and which were cytopathic for PHA cultures only. In previous experience, adenoviruses, herpes simplex, and vaccinia viruses were also isolated in this cell system (Jung and Matjašič, 1962).

SUMMARY

The sensitivity of PHA cell cultures for primary isolation of polioviruses has been investigated with materials of over 200 patients with paralytic poliomyelitis. The clinical

diagnosis could be confirmed by virus isolation in Slovenia, during 1960-1961, in 88,5 per cent of cases, ranging from 76,9 per cent in adults, up to 95,0 per cent in children aged 0 -4. Investigation of materials from the 1960 poliomyelitis epidemic in Croatia, performed by two laboratories, gave positive results in 78 and 81 per cent of cases under study, respectively. In Slovenia, only paralytic cases were found in paralytic cases, while in Croatia other enteroviruses could also be demonstrated. Results of comparative virus isolation attempts in PHA, HeLa and Detroit-6 cells were similar, as to poliovirus isolation; PHA cells were more sensitive for primary isolation of non-polio enteroviruses. Infectivity titrations of virulent and attenuated polioviruses in PHA and monkey kidney cells gave equally good results, while HeLa cells were less satisfactory.

PHA cell cultures were susceptible for primary isolation of Coxsackie B virus types 1, 3, and 5, Coxsackie A 9 and 23 (ECHO 9), and some unidentified group A strains, and ECHO virus types 1(8), 2, 3, 6, 7, 9 (Coxsackie A 23), and 16. More than a hundred of non-polio enteroviruses, of which most detected in healthy children, were not identified. In previous experience of this laboratory adenoviruses, herpes simplex, and vaccinia viruses could also be isolated in these cells.

PHA cells seem to be a useful and economical substitute for monkey kidney cells, since successful primary isolations of many cytopathic enteroviruses could be obtained.

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TABLE 1. PRELIMINARY GROUPING OF VIRUS ISOLATES BY MEANS OF TISSUE AFFINITY AND SUCKLING-MICE PATHOGENICITY TESTS

Viruses	Cytopathogenic effect in cultures of PHA	HeLa (§)	Embryonic pig kidney	Pathogenicity for suckling-mice
Polioviruses	+	+	0	0 (&)
Coxsackie B	+	+	+	+
Coxsackie A	0 (%)	0	0	+
ECHO viruses	+ (?)	0	0	0
Adenoviruses	+	+	0 (")	0

(§) HeLa cells, strain Zürich. The cell strain has undergone 160 passages in this laboratory during the past five years.

(&) Some type 2 strains may be pathogenic.

(%) Types 9 and 23 are positive. Strains of types 11, 13, 15 and 18 may also be cytopathogenic.

(?) Some types could not be propagated in these cells.

(") Some types may cause cytopathogenic changes.

TABLE 2. RESULTS OF ENTEROVIRUS ISOLATION IN SLOVENIA DURING 1958 - 1959 FROM PATIENTS WITH PARALYTIC POLIOMYELITIS AND ASEPTIC MENINGITIS IN VARIOUS TISSUE CULTURE SYSTEMS

Virus strains	PARALYTIC POLIOMYELITIS			ASEPTIC MENINGITIS		
	PHA (§)	HeLa	Detroit-6	PHA	HeLa	Detroit-6
Poliovirus type 1	17	16	16	1	1	1
type 2	6	6	6	1		1
type 3	1	1	1			
Unidentified (&)	2			2		
TOTAL	26	23	23	4	1	2

(§) Primary human amnion cell cultures.

(&) Cytopathic activity of the virus strains could not be neutralized by immune antisera pools against Polio 1-3, ECHO 1-14, Coxsackie A 9 and 23, Coxsackie B 1-5, and Adeno 1-7.

TABLE 3. SEASONAL INCIDENCE AND VACCINATION HISTORY OF PARALYTIC POLIOMYELITIS PATIENTS IN SLOVENIA DURING 1960 - 1961

Date	No. of cases reported	VACCINATED (§) No. of cases reported	No. of cases confirmed Polioviruses 1	2	3	Total	NON-VACCINATED No. of cases reported	No. of cases confirmed Polioviruses 1	2	3	Total (&)
1960/1	1						1				
2											
3											
4	2						2	1			1/2
5	2						2	1		1	2/2
6											
7	4	1					3	1			1/1
8	2						2	1			1/2
9	14	2	1		1	2/2	12	3	1	4	8/8
10	17	7	3			3/5	10	6		2	8/8
11	16	5	3		2	5/5	11	4		4	8/8
12	6	1	1			1/1	5	2		3	5/5
1961/1	4	1	1			1/1	3	2		1	3/3
2	4	1	1			1/1	3			2	2/2
3	3	2			1	1/1	1			1	1/1
4											
5	1						1				0/1
6-9											
Total	76	20	10		4	14/16	56	21		18	40/45

(§) Children were vaccinated with the Salk vaccine produced in 1957-1959 by Parke, Davis & Co., and in 1960 by the Institute of Hygiene of Serbia.

(&) Nominator means the number of patients, from which polioviruses could be isolated in PHA cell cultures; denominator means the number of patients, from which pathological specimens were sent for laboratory investigation.

TABLE 4. RESULTS OF POLIOVIRUS ISOLATION IN VARIOUS AGE GROUPS OF PATIENTS WITH PARALYTIC POLIOMYELITIS IN SLOVENIA DURING 1960 - 1961

Age group (years)	POLIOVIRUS ISOLATION IN TISSUE CULTURE (§) Positive (&)	Negative	Per cent positive	Not examined (?)
0 - 4	19	1	95,0	5
5 - 9	15	2	88,2	3
10 - 19	10	1	90,9	2
20 +	10	3	76,9	5
TOTAL	54	7	88,5	15

(§) Primary human amnion cell cultures were used for virus isolation attempts. Complement-fixation and neutralization test were performed with sera from majority of the patients.

(&) Virus strains were identified by neutralization tests with the type-specific poliovirus antisera.

(?) Patients were reported as to have paralytic poliomyelitis; pathological specimens were not sent to the laboratory.

TABLE 5. POLIOVIRUS TYPES IN VARIOUS AGE GROUPS OF PATIENTS WITH PARALYTIC POLIOMYELITIS IN SLOVENIA DURING 1960 - 1961

Age group (years)	POLIOVIRUS STRAINS Type 1	Per cent	Type 2	Per cent	Type 3	Per cent	Total poliovirus
0 - 4	12	63,1	1	5,2	6	31,6	19
5 - 9	11	73,3			4	26,6	15
10 - 19	4	40,0			6	60,0	10
20 +	4	40,0			6	60,0	10

TABLE 6. POLIOVIRUS STRAINS ISOLATED IN PRIMARY HUMAN AMNION CELL CULTURES FROM HEALTHY CHILDREN IN SLOVENIA DURING 1960 - 1961

Materials collected	No. of specimens	Positive (§) (Per cent)	Negative	POLIOVIRUSES No. of strains isolated	Per cent polioviruses from no. of specimens	from no. of enterovirus.
May-June 1960	483	70 (14,5)	413	4	0,83	5,7
Nov.-Dec. 1960	512	90 (17,5)	420	36	7,0	40,0
February 1961	498	23 (4,6)	475	19	3,8	82,6
TOTAL	1493	183 (12,2)	1310	59	3,9	32,2

(§) Total number of enteroviruses isolated in PHA cell cultures.

TABLE 7. RESULTS OF ENTEROVIRUS ISOLATION FROM HEALTHY CHILDREN AND CLINICAL PATIENTS WITH PARALYTIC POLIOMYELITIS AND ASEPTIC MENINGITIS IN SLOVENIA DURING 1960 - 1961

Materials collected	Virus strains cytopathogenic for primary (§) and continuous (&) cell cultures Polio 1	2	3	Coxsackie B type 3	Adeno	Unidentified (?)	Virus strains cytopathogenic for primary cultures
Healthy children							
May-June 1960	2		2	16	6	1	43 (+)
Nov.-Dec. 1960	15		21		3	2	49 (+)
February 1961	9		10	1			3 (")
TOTAL	26		33	17	9	3	95
Clinical patients 1.I.1960-31.V.1961							
Paralytic cases	31	1	22				
Non-paralytic	2				2		5 (")
TOTAL	33	1	22		2		5

(§) Primary human amnion cell cultures.
(&) HeLa cells, strain Zürich.
(?) Cytopathogenic activity of the virus strains could not be neutralized by immune antisera pools against Polio 1-3, ECHO 1-8, Coxsackie A 9 and 23, and Coxsackie B 1-5.
(+) Immunologic identification not completed.
(") Including one strain of ECHO virus type 1(8).
(") Including one strain of ECHO virus type 2, 3 and 1(8).

TABLE 8. RESULTS OF VIRUS ISOLATION IN PRIMARY HUMAN AMNION CELL CULTURES FROM PATIENTS WITH PARALYTIC POLIOMYELITIS IN CROATIA DURING 1960

Virus isolation	Paralytic cases	Non-paralytic cases	Total
Poliovirus type 1	33	3	36
type 2	3		3
type 3	2		2
ECHO virus type 1(8)	2		2
type 7	3		3
Coxsackie A 23	1		1
Adenovirus	2		2
Unidentified (§)	1		1
Negative	13	1	14

(§) Cytopathic activity of the virus strains could not be neutralized by immune antisera pools against Polio 1-3, ECHO 1-8, Coxsackie A 9 and 23, and Coxsackie B 1-5.

TABLE 9. TIME OF APPEARANCE OF MICROSCOPICALLY VISIBLE CYTOPATHIC ACTIVITY OF VIRUS STRAINS ISOLATED IN PRIMARY HUMAN AMNION CELL CULTURES

Virus strains	DAYS OF OBSERVATION (§)														Total
	1	2	3	4	5	6	7	8	9	10	11	12	15	17	26	
Poliovirus 1	1	10	5	13	2	1	4		2	2				1		43
2										3		1				4
3				1							1	1				3
ECHO		4	1													5
Adenovirus															2	2
Coxsackie A23												1				1
Unidentified(&)	1															1

(§) Cell cultures have been examined for 10 days; two blind passages were made if isolation attempt was negative.
(&) See table 8.

TABLE 10. INFECTIVITY TITRATIONS OF STANDARD ATTENUATED AND VIRULENT POLIOVIRUS STRAINS IN PRIMARY HUMAN AMNION, MONKEY KIDNEY, AND HELA CELL CULTURES INCUBATED AT 36°C AND 40°C

Virus strains	Virulence	$\log_{10}$ $TCID_{50}$ (§)					$\log_{10} \frac{TCID_{50}\,36°C}{TCID_{50}\,40°C}$	
		PHA		MK		HeLa		
		36°C	40°C	36°C	40°C	36°C	PHA	MK
Sabin type 1	0	7,0/2(&)	2,50/2	6,83/1	≤0,50/1	6,50/2	4,50/2	≥6,33/1
Sabin type 2	0	6,83/2	1,50/2				5,33/2	
Sabin type 3	0	6,61/3	2,17/3	6,50/1	1,50/1	3,50/1	4,44/3	5,00/1
Chat type 1	0	7,17/2	1,84/2			5,50/1	5,33/2	
Brunhilde type 1	+	7,09/4	6,50/4	7,10/1	6,50/1	6,50/2	0,59/4	0,60/1
E551-1105 type 2	+	7,50/2	6,83/2				0,67/2	
E543-4934 type 3	+	5,79/3	4,72/3	6,50/1	5,50/1	4,27/1	1,07/3	1,00/1

(§) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.
(&) Denominator means the number of titrations performed; nominator represents the median value of repeated experiments.

TABLE 11. INFECTIVITY TITRATIONS OF TYPE 1 POLIOVIRUS STRAINS IN PRIMARY HUMAN AMNION CELL CULTURES INCUBATED AT 36°C AND 40°C, AND IN HELA CELL CULTURES INCUBATED AT 36°C

Material (§)	Days after vaccination	$\log_{10}$ $TCID_{50}$ (&)			$\log_{10} \frac{TCID_{50}\,36°C}{TCID_{50}\,40°C}$
		PHA		HeLa	
		36°C	40°C	36°C	PHA
6572 RS	14	5,75	≤1,50	5,75	≥4,25
6573 RS	14	5,50	≤1,50	5,75	≥4,00
6588 RS	14	6,00	1,50	5,75	4,00
6601 RS	14	6,25	1,50	5,25	4,75
6609 RS	14	6,75	1,50	4,00	5,25
6620 RS	14	5,75	1,75	5,75	4,00
6624 RS	14	6,00	1,50	5,75	4,50
6396 RS	14	6,00	1,75	neg. (?)	4,25
6397 RS	14	6,25	1,25	neg. (?)	5,00
6640 RS	14	6,25	1,00	5,50	5,25
5080 F	27 (§)	5,00	3,00	5,00	2,00
5086 F	31 (§)	5,50	≥4,50	5,50	≤1,00

(§) Second tissue culture passage was used. Virus strains were isolated in PHA cell cultures from rectal swabs of children 14 days after they were fed the Sabin type 1 poliovirus vaccine. F = faeces
(&) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.
(?) In HeLa cells no CPE was demonstrated, $_{50}$ although 1,000.000 $TCID_{50}$ of the virus was used as inoculum.
(§) Paralytic poliomyelitis cases from 1960 epidemic in Croatia.

TABLE 12. INFECTIVITY TITRATIONS OF TYPE 1 POLIOVIRUS STRAINS IN PRIMARY HUMAN AMNION CELL CULTURES AND MONKEY KIDNEY CELLS INCUBATED AT 36°C AND 40°C

Material (§)	$\log_{10}$ $TCID_{50}$ (&)				$\log_{10} \frac{TCID_{50}\,36°C}{TCID_{50}\,40°C}$	
	PHA		MK			
	36°C	40°C	36°C	40°C	PHA	MK
6588	6,00	1,50	7,25	1,25	4,50	6,00
6601	6,25	1,50	6,25	≤0,50	4,75	≥5,75
6609	6,75	1,50	5,25	≤0,50	5,25	≥4,75
6620	5,75	1,75	7,25	≤0,50	4,00	≥6,75
6624	6,00	1,50	5,75	≤0,50	4,50	≥5,25
6396	6,00	1,75	5,50	0,75	4,25	4,75
6397	6,25	1,25	5,50	≤0,50	5,00	≥5,00
6640	6,25	1,00	6,50	1,50	5,25	5,00

(§) Virus strains were isolated in PHA cell cultures from rectal swabs of children 14 days after they were fed the Sabin type 1 poliovirus vaccine.
(&) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.

TABLE 13. INFECTIVITY TITRATIONS OF TYPE 3 POLIOVIRUS STRAINS IN PRIMARY HUMAN AMNION AND MONKEY KIDNEY CELL CULTURES INCUBATED AT 36°C AND 40°C

Material (§)	Clinical diagnosis	Days after type 3 vaccine	$\log_{10}$ $TCID_{50}$ (&)				$\log_{10} \frac{TCID_{50}\,36°C}{TCID_{50}\,40°C}$	
			PHA		MK			
			36°C	40°C	36°C	40°C	PHA	MK
5829 F	Aseptic meningitis	/	6,50	1,50	7,00	0,75	5,00	6,25
5829aF			4,75	2,00	4,75	2,75	2,75	2,00
6171 F	Entero-colitis	/	4,75	3,50	4,25	2,75	1,25	1,50
6180 F	Pertussis	25	5,75	4,00	5,00	4,50	1,75	0,50
6472 RS	Healthy	86	5,00	3,25	5,50	3,25	1,75	2,25
6564 RS	Healthy	86	5,00	2,50	5,75	4,75	2,50	1,00
6690 RS	Healthy	86	5,00	2,00	≤3,50	0,75	3,00	≤2,75

(§) Second tissue culture passage was used.
(&) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.

TABLE 14. INFECTIVITY TITRATIONS OF VIRULENT AND ATTENUATED POLIOVIRUS STRAINS IN PRIMARY HUMAN AMNION AND MONKEY KIDNEY CELL CULTURES INCUBATED AT 36°C AND 40°C. AFTER 7 DAYS THE CULTURES AT 40°C WERE ADDITIONALLY INCUBATED AT 36°C FOR 72 HOURS

Poliovirus strain	Type	$\log_{10}$ $TCID_{50}$ (§)					
		P H A			M K		
		36°C	40°C	36(40)°C	36°C	40°C	36(40)°C
Chat	1	7,17	2,17	2,17			
Sabin	2 (&)	6,83/2	1,50/2	1,50/2			
Sabin	3				6,50	1,50	2,75
E551-1105	2	7,50/2	6,83/2	6,83/2			
E543-4934	3				6,50	5,50	5,50
6568	1	6,00	1,50	1,50			
6601	1	6,25	1,50	1,50			
6375	2	5,75	2,25	2,75			
6391	2	6,00	≥4,50	≥4,50			
6467	2	6,25	2,75	3,00			
6468	2	6,50	2,25	2,50			
6492	2	7,25	2,25	2,25			
6493	2	6,25	2,50	2,75			
6508	2	5,25	1,50	2,75			
6523	2	6,75	2,73	3,00			
6529	2	6,27	1,00	2,00			
6539	2	5,75	1,75	2,75			
5829	3				7,00	0,75	1,00
6171	3				4,25	2,75	3,00
6179	3	6,25	4,25	4,50			
6180	3	5,75	4,50	4,50	5,00	4,50	4,50
6181	3	5,75	≥4,50	≥4,50			
6342	3	4,75	2,00	2,00	4,75	2,75	2,75

(§) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.
(&) Denominator means the number of titrations performed; nominator means the median value of repeated experiments.

TABLE 15. INFECTIVITY TITERS OF SOME ECHO VIRUS STRAINS ISOLATED IN PRIMARY HUMAN AMNION CELL CULTURES

Virus type	Virus strain (§)	$\log_{10}$ $TCID_{50}$ (&)
ECHO 1(8)	7135	5,5
	7580A	6,0
	10063	7,0
	10091	7,0
	10400	7,0
ECHO 6	1535	6,0
	6546	5,0
	9831	7,5
	10744	5,0
ECHO 9	7205	7,0
	7206	6,5
ECHO 16	9634	5,5
	10440	5,0

(§) Second tissue culture passage.
(&) Infectivity titers expressed as log $TCID_{50}$ per 0,1 ml.

TABLE 16. PRIMARY ENTEROVIRUS ISOLATIONS IN PRIMARY HUMAN AMNION CELL CULTURES FROM PATIENTS WITH ASEPTIC MENINGITIS IN CROATIA AND SLOVENIA DURING 1962

	ECHO viruses			Coxsackie B			Coxsackie A		Unidentified (§)	Not typed
	1(8)	6	16	1	3	5	9	not typed		
Croatia				12	4	1	2			7
Slovenia	3	1	2			1		1	7	11
Total	3	1	2	12	4	2	2	1	7	18

(§) Cytopathic activity of the virus strains could not be neutralized by immune antisera pools against Polio 1-3, ECHO 1-8, Coxsackie A 9 and 23, and Coxsackie B 1-5.

STAT

CULTIVATION OF VACCINIA VIRUS IN HUMAN DIPLOID CELL STRAINS

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Numerous tissue cultures of the most diverse origin are sensitive to the virus of vaccinia. In view of the presumed suitability of human diploid cell strains for the preparation of antiviral vaccines it was interesting to ascertain if these cells are sensitive to the vaccinia virus and what height of titre the virus attains in them as compared with other cell systems.

Material and Methods

Diploid cell strains WI-26 and WI-38 obtained from Dr Hayflick of the Wistar Institute in Philadelphia were used in the experiments. The WI-26 strain was used in its 24th to 43rd passage, the WI-38 strain in its 21st to 31st passage. Eagle's medium with 10% of calf serum and 1/3 of 0.5% LAH in Hank's solution was used as the growth medium. The maintenance medium after inoculation of the tissues was incomplete Parker medium 199 in a modification of the Serum and Vaccine Institute, Prague /Čs. Epid. Mikrobiol. Imunol., 2, 11, 1960/, with 2% of calf serum. The test tube cultures were set up from a cell suspension containing 100,000 cells per ml and test tube.

Parallel experiments with primary tissue cultures from Macaccus rhesus kidney /OL/ and from dog kidney /PL/ were carried out. Modified Parker medium 199 /as above/ with different quantities of calf serum was employed as growth medium. The density of the seeding suspension was 100,000 cells per ml and test tube for the monkey-kidney primary cultures and approximately 200,000 cells per ml and test tube for the dog kidney primary cultures. Modified Parker medium 199 with 2% of calf serum was used as maintenance medium in both types of tissue culture.

Vaccinia virus strains E-4 /ID_{50}/0.2 ml for the CAM equalled $10^{-4.3}$/ and 20 E-2, ovovaccine /ID_{50}/0.2 ml for the rabbit equalled $10^{-3.7}$/ obtained from the Serum and Vaccine Institute were used for inoculating the cells.

Virus was titrated from the cytopathogenic effect, pronounced though focal destruction being taken as the endpoint. Four to five test tubes were used for each virus dilution. The tissue cultures were observed for 10 days following inoculation. Bteween the passages the virus was stored on dry ice or in a Revco ice-box at -60°C.

The $TCID_{50}$ was calculated by the Reed-Muench method.

Results

The vaccinia virus produces a pronounced cytopathogenic effect in human diploid cell strains. The destruction of the tissue consists in a clustering of the cells and in a retraction and disintegration of the cytoplasm, which bridges by narrow strips the vacancies in the tissue joining the cell clusters. The clusters consist of deeply stainable pathologically changed nuclei and cytoplasm debris. A higher virus dilution causes only a focal cytopathogenic effect.

In the WI-26 diploid strain five passages of the vaccinia strain E-4 and four passages of the 20 E-2 ovovaccinum variolae strain were performed /Table 1/.

Table 1

Experiment No	Material Inoculated	Tissue	Virus dilution	Log.$TCID_{50}$/0.1 ml 1st passage	2nd	3rd	4th	5th
1	Strain E-4	WI-26 31st-36th passage	concent.	-6.5	-4.7	-4.2	-4.2	-4.5
2	Ovovaccin. variolae 20 E-20	WI-26 37th-43rd passage	10^{-2}	-6.5	-5.6	-6.3	-5.6	-

In the WI-38 diploid cell strain twice four passages of the ovovaccinum variolae 20 E-2 strain were performed /Table 2/.

Table 2

Experiment No	Material inoculated	Tissue	Virus dilution	Log. $TCID_{50}$/0.1 ml 1st	2nd	3rd	4th passage
1	20 E-2	WI-38 19th-23rd passage	10^{-2}	-3.5	-6.4	-5.5	-7.0
2	20 E-2	WI-38 27th-31st passage	10^{-2}	-5.6	-5.2	-5.5	-6.0

The virus titre did not increase by passaging virus diluted 10^{-1}, 10^{-3}, 10^{-4} /log.$TCID_{50}$/0.1 ml under these conditions equalled -5.5 and -6.0/.

An approximately equivalent sensitivity of the WI-26 and the WI-38 strains to the vaccinia virus was demonstrated by the cultivation and titration of the virus under identical conditions in both strains /Experiments No 2 in Tables 1 and 2/.

Furthermore, comparative titrations of the vaccinia virus in the diploid cell strains and in primary tissue cultures from monkey and dog kidney were carried out /Tables 3 and 4/.

Table 3

Experiment No	Material inoculated	Virus dilution	Tissue	Log. $TCID_{50}$/0.1 ml 1st passage	2nd passage
1	20 E-2	10^{-1}	OL	-4.6	-5.1
2	20 E-2	10^{-2}	WI-38 30th p.	-4.5	-6.5
			OL	-5.3	-5.2
3	E_4		WI-26 25th p.	-5.5	
			OL	-6.2	
4	E_4		WI-26 29th p.	-5.6	
			OL	-4.6	

The above comparison of titre of the vaccinia virus in first passages in diploid cell strains and in primary monkey kidney cultures shows that the sensitivity to the virus of both cell systems is more or less the same.

Table 4

Experiment No	Material inoculated	Virus dilution	Tissue	Log.$TCID_{50}$/0.1 ml 1st	2nd	3rd	4th passage
1	E_{20}	10^{-2}	PL	-4.8	-5.4	-5.0	-4.2
2	E_4		WI-26 29th p.	-5.6			
			PL	-5.3			

No substantial differences between the titres of vaccinia virus obtained in the first passages in human diploid cell strains and primary dog kidney cultures was noted.

It was found in two experiments that the titre of vaccinia virus cultivated in the human diploid strains WI-26 and WI-38 is not changed by storage on dry ice for three to eight months.

Discussion

The above results show that human diploid cell strains are highly sensitive to vaccinia virus. Should these tissues prove of use for the production of antivriral vaccines, the possibility of utilizing them for the preparation of an anti-variola vaccine could be considered.

We are continuing our comparative studies on the sensitivity og human diploid cell strains and other systems and on the stability of virus in long-term cultivation and storage.

Summary

A hight sensitivity to the vaccinia virus was observed in human diploid cell strains and was compared with the sensitivity of primary monkey kidney and dog kidney cultures.

V/6

UTILIZATION OF A NEW DIPLOID CELL STRAIN DERIVED FROM HUMAN EMBRYO LUNG TISSUE FOR THE CULTIVATION OF ENTEROVIRUSES AND MEASLES-VIRUS

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Diploid cell strains from the very beginning adapt to the composition of the nutrient medium, and the success of their maintenance in passages is, apparently, to a considerable degree dependent on the identity of cultivation conditions and the conditions of primary culture. A serious drawback in the use of primary Hayflick-Moorhead diploid strains is their marked adaptability to the amino acid complex manufactured by the Microbiological Associates Company. The experience of many researchers, our own included, has been that the replacement of that complex by amino acids of a different origin results in the death of the strain.

An attempt has been made to obtain our own diploid strains on available media. After trials with a number of media we chose a mixture of 70 parts of "Igla" medium /of amino acids of the California Corporation for Biochemical Research and of Czechoslovak-made Chemerol, vitamins produced by NBC/ and 30 parts of a 0.5% solution of lactalbumine hydrolysate in Hanks medium+. The "Igla" medium contained 10% of calf serum.

Using Hayflick and Moorhead techniques /1,2/, we obtained five cellular strains from the lung tissue of human embryos. Our paper presents the materials concerning research into one of them, which was passed through 48 subcultivation passages /strain LT-16/.

+Thnaks to personal communication from Dr J.Trlifajová /I.E.M. Prague/ we were aware of the successful use of lactalbumine hydrolysate, to supplement the "Igla" medium /prepared from amino acid and vitamins by Microbiological Associates Co/ in the culture of original Hayflick and Moorhead strains.

The object of the first part of our research was to show the correspondence of the charcateristics of our strain with the features of original Hayflick and Moorhead strains. The principal of these characteristics were in our opinion the dynamism and morphology of growth, intensity of cellular metabolism, karyotypic picture, and absence of oncogenicity in the experiment.

Dynamism of growth of LT-16 strain. The strain passed through three stages of development: formation /primary culture, 1-2 passages/, active growth /up to 43-45 passages/, and ageing. In the stage of active growth, as early as 24 hours after transplanatation of the culture, the protruding cells formed a net, merging into a monolayer after 48 hours of cultivation. After 74-98 hours, an at least twofold cellular layer was observable, oriented in intercrossing directions. Subcultivation passages were made twice weekly, with the growth surface being doubled. By the end of each subcultivation period /.i.e. after 3-4 days of growth/ the cell count in a one-litre matrix flask reached 18-24 millions. There was a decline in the intensity of growth during the stage of ageing and after 48 passages mitotic activity was discontinued.

Morphology of LT-16 strain. In the primary culture the cells had a predominantly epithelic form /Fig.1,a/. Three or four passages later they attained a fibroblastic form, took parallel positions and were characterized by monomorphism /Fig.1,b/. Cells mainained in passage for 10-12 days formed a multilayer membrane at the end of this period /Fig.1,b/. In the stage of ageing the cells assumed a loose arrangement, at times amorphous. Parellel with protruding elements a considerable number of cells was observable with oversize hyperchronic nuclei, sometimes amorphous, and with voluminous cytoplasm /Fig.1, /. Fluorescent microscopic examination revealed a considerable RNK content in cells of LT-16 strain. The differentation of cells was effected by alterations in RNK content.

Intensity of cellular metabolism, expressed trough acidification of the nutrient medium, was considerably enhanced in subcultivations and exceeded that commonly observable in cell

cultures of monkey kidney or inoculated lines. LT-16 cells preferably grew in media with a comparably low RN index.

Karyotype. Cells of LT-16 strain had the female diploid chromosome arrangement, common in man. Fig.2,a shows a cell in metaphase. The chromosomes of the same cell, arranged in groups according to Denver classification, are shown in Fig. 2,б No conspicuous quality alterations were observed in the idiogram. Counts acrried through with 200 cells undergoing metaphase revealed 8 /4%/ heteroploid cells in the 12th passage, 6 /3%/ in the 21st passage, and 2 /1%/ in the 34th passage. The decrease in number of heteroploid cells in the process of cultivation suggests the diploid stability of the LT-16 strain.

Oncogenicity. In the inoculation of hamster cheek pouches cells of LT-16 strain caused no increasing swelling. Between the 4th and 8th day following inoculation, very small /millet-grain-sized/ infiltrations were observed, which were resorbed after a few days. Histological research revealed that these infiltrations represented an ordinary inflammatory reaction developed at the site of induction of LT-16 cells /Fig.3/.

The charactetistics outlined allow, in our opinion, to consider LT-16 strains to be close in their qualities to Hayflick and Moorhead strains. Our task was to study the sensitivity of the new diploid strain to cerrain viruses.

It is known that somewhat lower titres of enteroviruses are obtained in diploid cell cultures. This is explained by the adaptability of laboratory virus strains to monkey cells /2/.

We checked titres of attenuated Sabin polioviruses on various levels of passage in LT-16 culture. It was revealed that during adaptation to the new culture the polioviruses grew no less intensively than in the cells of monkey kidney /Table 1/.

ECHO viruses of types 1-19, 20, 21, 26, 27 as well as Coxackie A_9 were cytopathogenic for LT-16 strain, but in titres less than 1 $\log_{10}$ in monkey kidney cultures /the possibility of adaptation has not been checked/. Coxackie B_{1-5} viruses caused no cytopathogenic alterations in our cell culture.

Table 1

Titres of Polioviruses in LT-16 Culture

Virus adapted in tissue culture	Titres after CPE /log TCD_{50}/ml in tissue culture of monkey kidney: types			in LT-16 culture: types		
	I	II	III	I	II	III
Monkey kidney	7.0	6.5	7.0	5.8		
LT-16 /1st passage/	7.0	6.5	8.0	6.6		
LT-16 /4th passage/				7.0	6.5	6.2
LT-16 /7th passage/				7.0		
LT-16 /9th passage/				7.4	7.0	8.0

The strains of measles viruses, Edmonston and Leningrad-4, were successfully adapted in LT-16 culture, causing cytopathogenic alterations. In the process, the virulent variant of Edmonston strain and the vaccinia strain Leningrad-4 caused the formation of oversize multinuclear cells, whereas the cytopathogenic effect of the attenuated variant of Edmonston strain was limited to the occurrence of a general cellular degeneration and dilution of cellular layer /Fig.4, a, б, в /. Fig.5 shows the dynamism of growth of virulent Edmonston strain by titres in LT-16 culture. Leningrad-4 strain was reproduced in this culture by approximately identical titres.

Conclusion

1. In the process of adaptation of polioviruses to a new strain of LT-16 diploid cells, the viruses were grown in this culture up to titres comparable with those commonly obtained in the cell culture of monkey kidney.
2. The virulent variant of the strain of Edmonston measles virus and the vaccinal measles virus Leningrad-4 gave rise to the formation of symplasts in LT-16 cell culture; upon infection by the attenuated variant of Edmonston strain, a degeneration of cells of LT-16 culture, with no formation of symplasts, was observed.

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