SUSCEPTIBILITY OF HUMAN AMNION (FL) CELL LINE TO THE ECHO VIRUSES

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Betsey Jane Krueger

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SUSCEPTIBILITY OF HUMAN AMNION (FL) CELL LINE TO THE ECHO VIRUSES

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Betsey Jane Krueger

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INTRODUCTION

The purpose of this investigation was to determine the susceptibility of the human amnion (FL) cell line to the enteric cytopathogenic human orphan (ECHO) viruses. The study was undertaken with two purposes in mind: (1) to determine if the FL cell strain would be suitable for the routine isolation of enteric viruses and (2) to further characterize the ECHO viruses.

The use of a continuous cell line, such as the FL strain, has several advantages over the use of a primary culture, such as monkey kidney, for virus isolation. Perhaps one of the major disadvantages in the use of primary cultures is that they often have been found to contain contaminating viruses from the source animals. Hull *et al.* (1956) and Brown (1957) have reported on spontaneously occurring simian viruses from tissue cultures of monkey kidney cells. Another advantage of the continuous cell culture is that it is generally easier to maintain and least expensive from the laboratory standpoint. Also, its use eliminates repetition of time-consuming preparation of tissues for primary cell cultures. Primary cell cultures exhibit varying susceptibility to the same viruses due to variations in source animals while continuous cultures usually do not vary as much in susceptibility. Therefore, it can be seen that a continuous cell line similar in susceptibility to the same viral spectrum as the primary monkey kidney culture would be most desirable.

Recently Fogh and Lund (1957) described the continuous cultivation of cells derived from a normal human amniotic membrane, which they call the FL strain. The susceptibility of the FL strain to the ECHO viruses was investigated to determine if this cell line, with all the inherent advantages of a continuous culture, would be susceptible to a portion of the viral spectrum of primary monkey kidney cultures.

I. The Enteric Cytopathogenic Human Orphan (ECHO) Viruses

The discovery by Enders, Weller, and Robbins (1949) that poliomyelitis viruses would multiply in tissue cultures of human embryonic tissue and the subsequent report by the same group of men, Robbins, Enders, and Weller (1950), that polioviruses caused cell injury and death (cytopathogenic effect) in tissue cultures provided the criteria by which the presence of viruses could be recognized in vitro. These discoveries led to the widespread use of various types of tissue cultures in all phases of poliomyelitis research. Among the different aspects of this research was the use of rhesus monkey kidney tissue cultures for screening of fecal specimens from patients with suspected poliomyelitis.

It soon became evident that among the polioviruses that were being isolated in tissue cultures other viruses were also present. Robbins et al. (1951) in a study on the direct isolation of virus strains from patients with non-paralytic and paralytic poliomyelitis found cytopathogenic agents that could not be classified among any of the known viruses. Melnick (1953) reported 10 agents, other than polioviruses, that were isolated from human stool specimens using monkey testicular tissue cultures. None of the ten was neutralized by poliomyelitis virus antisera. Three of these agents were pathogenic for suckling mice, which would place them in the Coxsackie group of viruses, the other seven exhibited no mouse pathogenicity.

In another publication, Melnick (1954) reviewed unpublished data on the isolation of these unclassified agents by various investigators and referred to them as "orphan viruses".

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Ramos-Alvarez and Sabin (1954) isolated 31 monkey kidney tissue culture cytopathogenic agents from 1566 rectal swabs of healthy children. Of these 31 agents, 5 were polioviruses, one was a Group B Coxsackie virus, and 25 belonged to a group of viruses they called the "human enteric viruses".

Horstmann (1955) while surveying endemic virus infections in Egypt found 113 of 319 infants infected with some agent which exhibited cytopathogenic effects in monkey kidney tissue cultures. Fourteen of these children were excreting polioviruses, eight Coxsackie viruses, and 91 others "orphan viruses".

In 1955 a committe consisting of G. Dalldorf, J. F. Enders, W. McD. Hammon, A. B. Sabin, J. T. Syverton, with J. L. Melnick as chairman, working under the auspices of the National Foundation for Infantile Paralysis, reported on various aspects of this new group of viruses (Committee on the ECHO Viruses, 1955). The committee was formed to evaluate the significance of these new viruses. This group of viruses which had previously been called "orphan viruses" and "human enteric viruses" were now named the "enteric cytopathogenic human orphan (ECHO) viruses".

The ECHO group of viruses were isolated through the use of tissue culture techniques from fecal specimens of patients with the clinical syndrome of aseptic meningitis, from healthy children in various areas of the world (the Philippine Islands, Egypt, and the United States), and during surveys of epidemics occurring in the poliomyelitis season.

The committee reported the recognition of 13 antigenically distinct ECHO viruses sharing certain properties. All were isolated in monkey kidney tissue cultures and were cytopathogenic for both simian and human cell cultures. Antisera against the three poliovirus types, Coxsackie group B and Coxsackie group A type 9 viruses did not neutralize their cytopathogenic effect on tissue cultures. They produced no disease in suckling mice nor in other small laboratory animals. The committee also reported that they were not related to other viruses recovered from the alimentary tract by tissue culture techniques. The ECHO viruses are neutralized by individual human serum and by pooled human gamma globulin.

The ECHO group is further characterized in the committee's report as follows:

> Other studies of the ECHO viruses (more extensive for some than for others) have provided additional information. Complement-fixing antigens have been detected in the culture fluids of a number of viruses that have been tested. All the viruses tested were ether-resistant. Ultrafiltration (gradocol membrane) measurements indicated sizes for types 1, 2, and 3 between 11 and 17 mμ. The size of type 10 is reported to be between 60 and 90 mμ. Plaque morphology of the ECHO viruses studied (types 1, 3, 4, 5, 6, 7, and 9) is sufficiently distinctive, except for type 7 (Garnett strain), to permit differentiation from polio virus plaques. The plaques of the ECHO viruses mentioned had irregular diffuse boundaries, and healthy cells could be found within the degenerated areas.
>
> Kidney cells of different monkey species vary in their susceptibility to the ECHO viruses. Rhesus (Macaca mulatta) and cynomolgus (M. irus) cells are susceptible to all 13 types studied. Cells from the South American capuchin (Cebus capucina) were found to be resistant to types 1, 2, 3, 7, 8, 9, and 11. However, they were susceptible to type 10. Cells from the African red grass military monkey (Erythrocebus patas), which were resistant to types 1, 2, 3, 4, 5, 6, and 9, were as susceptible as those from the rhesus monkey to the type 7 Garnett strain. (Committee on the ECHO Viruses, 1955, page 1188)

In 1957, the name of the Committee on the ECHO Viruses was changed to the Committee on the Enteroviruses (1957) to include in its consideration not only the ECHO viruses but also the polioviruses and the Coxsackie viruses, Groups A and B. The committee reported that six new ECHO antigenic types had been recognized and confirmed thus bringing the number of ECHO viruses to 19.

Other investigators have reported various properties of the ECHO group since the first report by the Committee on the ECHO Viruses (1955). Lerner et al. (1957) reported on the susceptibility of primary human chorion cells to ECHO types 1 - 14. They found that ECHO viruses 1, 3, 4, 6, and 11 produced rounding of the epithelial cells while sparing many of the fibroblasts in the cultures. ECHO viruses 2, 7, 8, 9, 10, 12, 13, and 14 produced no cytopathic changes in the cultures and did not multiply.

In comparing the behavior of ECHO types 1 - 16 in fibroblast-like and epithelial-like human cell strains, Stulberg, Page, and Berman (1958) found that all the ECHO types (except type 10) multiplied in the fibroblast-like cell line. Of five epithelial-like cell strains that were tested the ECHO viruses either did not grow at all or only a few types grew in the various strains.

Archetti, Weston, and Wenner (1957) found that ECHO types 1 through 13, with the exception of type 4, were adaptable to HeLa cell cultures with cytopathogenic effect to the cultures. They also reported that the material from the 5th passage in HeLa cultures gave specific reliable antigens for use in the complement fixation test.

Using tissue cultures from human amniotic membranes, Lahelle (1957) found that these primary cultures were more susceptible to ECHO virus type 6 (1 - 2 log units higher) than monkey kidney tissue cultures.

Studying the effect of hydrogen ion concentration on the cytopathopathogenicity of ECHO viruses, Barron and Karzon (1957) found that the cytopathogenic titer of certain ECHO viruses in monkey kidney tissue cultures was depressed or delayed when grown in the presence of a medium that developed an acid pH.

The ECHO viruses have not been connected with any specific diseases at this time. However, ECHO viruses types 6 and 9 have been incriminated as the etiologic agent in cases of aseptic meningitis.

Davis and Melnick (1956) reported the isolation of ECHO type 6 from 24 cases of aseptic meningitis. Karzon et al. (1956) have also reported the isolation of ECHO type 6 from seven cases of aseptic meningitis. It was stated by Davis and Melnick (1956) that " . . . the present report of echo virus type 6 as the only agent associated with 24 cases of the disease lends strong support to the view that this virus is one of the etiological agents of aseptic meningitis".

Rhodes and Beale (1957) also incriminate some of the ECHO viruses, along with Coxsackie group B viruses and other viruses, among the viral causes of aseptic meningitis.

Faulkner et al. (1957) isolated an agent, later identified as ECHO type 9, from stool specimens (and cerebrospinal fluid, in one instance) of 3 patients with aseptic meningtis.

ECHO virus type 9 was found to be the etiological agent in an epidemic of aseptic meningitis which occurred in Western Europe (Quersin-Thiry et al., 1957). Partly on the basis that this agent was pathogenic for suckling mice, they proposed that ECHO virus type 9 be reclassified as a member of the Coxsackie group A viruses. (The ECHO virus type 9 isolated by Faulkner et al. also was pathogenic for suckling mice.)

II. Human Amnion Cell Cultures

The continuous human amnion cell strain (FL) as developed by Fogh and Lund (1957) is composed of epithelial-like cells. The cell line was started from a normal human amniotic membrane by trypsin digestion in November 1956 and has been cultivated in serial passage since that time.

Beyond the original report of the continuous cultivation of cells from the amniotic membrane by Fogh and Lund no subsequent reports on the use of this cell line for virus propagation have appeared. However, there have been several reports on the use of primary amniotic membrane cell cultures for virus propagation. In the hope that characteristics of the primary cultures are similar to the continuous cultures the findings of several investigators are summarized.

Lahelle (1957), Weinstein (1956), Dunnebacke (1956), and Wilt (1956) have all found that human amnion cells are susceptible (as reflected by cytopathogenic changes) to the three types of polioviruses.

Dunnebacke (1956) reported that human amnion cells differed from HeLa, monkey kidney, and human fetal cells in their cellular changes when infected with the polioviruses. The virus was released approximately 48 hours later in amnion cells. Other differences concern the nucleolus and the formation of small nodules on the cell's surface.

Takemoto and Lerner (1957) found that primary human amnion cell cultures were susceptible to the adenoviruses types 1 - 8, Group A-9 Coxsackie virus, types B1, B3, and B5, and one of four B2 strains of Coxsackie viruses, and herpes simplex virus. Weinstein (1956) found that Coxsackie type B3 produced cytopathogenic effect but Group B types 2, 3, and 4 did not. In addition the primary amnion cell cultures were susceptible to Coxsackie Group A type 9, herpes simplex, and western equine encephalomyelitis viruses but not influenza viruses strains A and B.

Infectious bovine rhinotracheitis (Cheatham and Crandell, 1957) and measles (Milovanovic et al., 1957) viruses have also been grown in primary human amnion cultures.

Primary amnion cultures are reportedly more susceptible to polioviruses and ECHO viruses than monkey kidney cultures (Lahelle, 1957). Using 4 strains of ECHO virus type 6, Lahelle obtained titers 1 - 2 log units higher when the virus was propagated in amnion cell cultures than when it was grown in monkey kidney cultures.

Comparing the relative usefulness of primary amnion cultures for the routine isolation of enteric viruses, Kelly (1957) found that they were not as satisfactory as a combination of monkey kidney cells and suckling mice but better than HeLa or Detroit-6 cells as determined by the number of virus isolations from each system.

MATERIALS AND METHODS

I. Cleaning and Preparation of Glassware

All equipment (glassware, filters, pipettes, etc.) was washed in a warm solution of Haemo-sol (Meinecke and Co., New York, N. Y.), rinsed 6 times in tap water, 6 times in distilled water, and once in glass-distilled water. The glassware was sterilized by dry heat at 300° F for three hours. Other equipment was sterilized in the autoclave at 15 lbs pressure (121° C) for 15 minutes.

II. Media

The nutrient medium used to maintain the monkey kidney epithelial cells was made from 10X concentrate synthetic Medium 199 (Morgan, Morton, and Parker, 1950) with 2% inactivate horse serum. The hydrogen ion concentration of the medium was adjusted to a range of 7.0 to 7.2 with 2.8% $NaHCO_3$. This medium will be referred to as M-199.

The nutrient medium used for the cultivation of the amnion cell line consisted of Eagle's (1955) basal medium (BME) with 20% inactivated human serum. For virus propagation BME with 5% inactivated calf serum was used .

The components of Eagle's basal medium and synthetic mixture 199 were obtained from Microbiological Associates, Inc., Bethesda, Maryland.

Glass-distilled water was used to make up all media. To avoid bacterial contamination 100 units of penicillin and 100 micrograms of streptomycin were added to each ml of medium.

Human, horse, and calf sera were obtained from normal animals by allowing the blood to clot and removing the serum. The serum was centrifuged at 2000 rpm for 10 minutes in an International Centrifuge, size 2, Model V, to remove any red blood cells, inactivated by heating in a water bath at 56° C for 30 minutes, and tested for bacterial sterility. Before routine use all sera were checked in tissue culture to determine if they were free from toxic properties and then stored at -20° C until they were used.

The trypsin solution used for dispersion of the amnion cell cultures was prepared from Bacto-Trypsin 1:250 (Difco Corp., Detroit, Michigan) in a concentration of 0.25% by weight using calcium free Hank's balanced salt solution as diluent. The solution was sterilized by Seitz-filtering under negative pressure.

III. Tissue Culture

Monolayer cultures of rhesus monkey kidney epithelial cells, used for titration of the infectivity of the viruses, were purchased from Microbiological Associates, Inc. These were shipped by air express in 16 x 150 mm screw-cap tubes ready for use. After arrival the medium was replaced with 0.5 ml M-199 and the tubes were incubated at 37° C for at least 24 hours before inoculation with the viruses.

The original culture of the continuous amnion (FL) cell line was received from The Carver Foundation, Tuskegee Institute, Alabama.

Stock cultures of the amnion cell line were cultivated in 8 ounce prescription bottles. Cultures were transferred weekly in the following manner: the nutrient fluid was removed from the cell layer and replaced by an equal volume of 0.25% trypsin solution. The trypsin solution remained on the cell layer until the cell sheet was loosened and floated in the solution. This required about 5 to 10 minutes at room temperature. With a pipette the trypsin solution, containing the cells in suspension, was transferred to a 15 ml conical centrifuge tube. The solution was drawn into and dispelled from the pipette several times to break up clumps of cells. The cell suspension was then centrifuged at 600 rpm for 10 minutes. The supernatant fluid was removed and the cells were resuspended in 5 ml of BME containing 20% inactivated human serum. At this time a count of the cells was made.

One part of the FL cell suspension was mixed with nine parts 0.1% crystal violet in 0.1M citric acid to facilitate counting. A hemocytometer was used to determine the number of cells in suspension. Calculations were made according to the method presented in Diagnostic Procedures for Virus and Rickettsial Diseases (1956). The cell suspension was then diluted to contain 100,000 cells/ml and transferred into new culture bottles. Ten ml of the suspension were placed in each culture bottle.

At first, culture tubes (16 x 150 mm) were made using one ml of a suspension containing 50,000 cells/ml. When it was found that a good monolayer of cells was not obtained using this concentration of cells it was increased to 75,000 cells per tube. This concentration produced a better monolayer of cells and was used thereafter.

Both culture bottles and tubes were incubated at 37° C in a horizontal, stationary position.

Nutrient medium was replaced in the culture bottles every 48 - 72 hours. At the end of 5 or 6 days a layer of cells covered the side of the bottle. On the seventh day the cultures were trypsinized and transferred.

The culture tubes were not disturbed for 6 days. At the end of this time the fluid was removed, the cells were washed three times with Hank's balanced salt solution and 0.5 ml fresh BME containing 5% inactivated calf serum was added to the tubes. The tubes were examined under the low power of the microscope and those tubes with good cellular growth were inoculated with viruses.

IV. Virus Strains

Nineteen prototype ECHO viruses were investigated. ECHO viruses types 1- 6 and 14 were obtained from the American Type Culture Collection, ECHO viruses types 7 - 13 and 16 were received from Dr. Stulberg, Child Research Center, Detroit, Michigan, and ECHO viruses types 15 and 17 - 19 were received from Dr. Wenner, University of Kansas, Kansas City, Kansas. Three prototype polioviruses, type I (Mahoney), type II (MEF-1), and type III (Saukett) were used as positive controls to infect the amnion cell cultures. All viruses were stored at -20° C.

V. Monkey Kidney Tissue Culture Inoculation

Titrations of the ECHO viruses were performed in the susceptible monkey kidney tissue cultures to determine the infectivity titer (TCD_{50}) of the viruses. Ten-fold dilutions of the individual viruses were made in M-199. Each dilution was inoculated into 3 tubes of monkey kidney cells using 0.1 ml per tube. The tubes were examined daily for 10 - 12 days after inoculation for microscopic signs of cellular degeneration produced by the virus. Media were replaced on the culture tubes every 3 or 4 days. The fifty-percent endpoints were calculated by the method of Reed and Muench (1938) and expressed as TCD_{50}/ml. Each titration was done twice.

VI. FL Cell Strain Inoculation

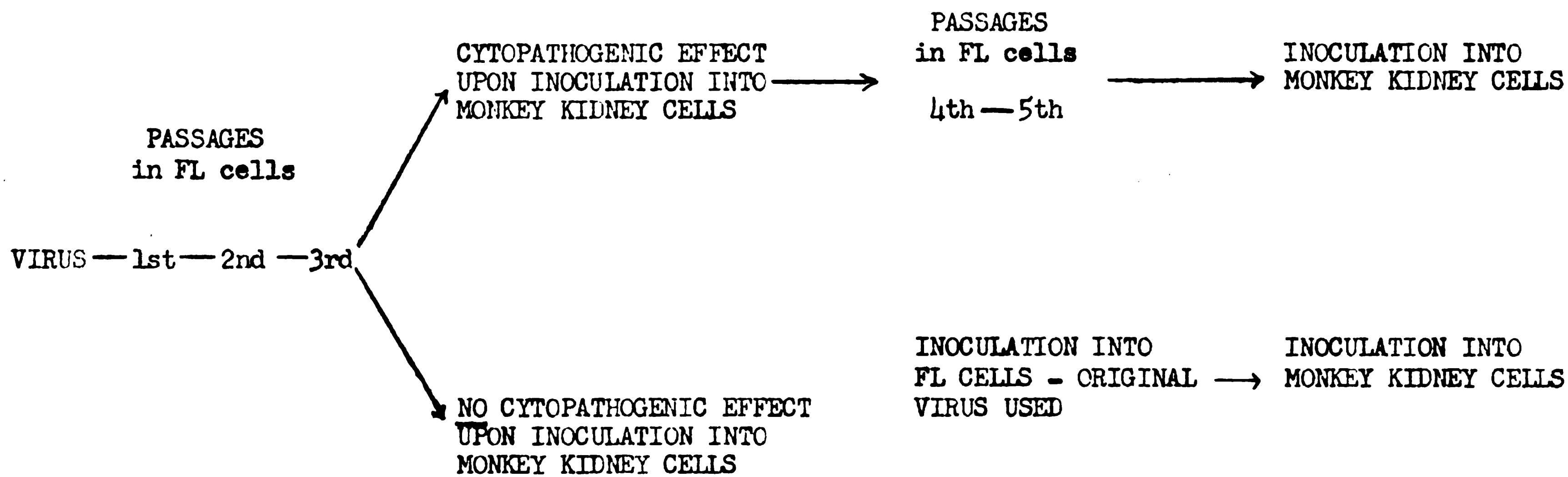
FL cell strain cultures were inoculated with 0.1 ml per tube of undiluted or 10^{-1} dilutions of the respective ECHO and polio viruses. As calculated from the infectivity titers of the viruses the undiluted and 10^{-1} dilutions contained from 10^3 to 10^8 tissue culture infecting doses per 0.1 ml. The exact inoculum used of each virus is presented in Table II. Each virus was inoculated into three tubes (0.1 ml per tube) of FL cells and observed for cytopathogenic effect. The medium in the tubes was replaced every three or four days. The medium that was removed from each set of three tubes was pooled and frozen. All medium from the respective virus inoculations were pooled separately to be used for further inoculations or for blind passages.

If no cytopathogenic effect was observed blind passages were made. (See Figure I for a schematic presentation of FL cell strain inoculations with the ECHO viruses). This was done by the inoculation of 0.1 ml per tube of the pooled medium into another set of 3 FL culture tubes. Blind passages were performed to get a maximum expression of cytopathogenicity of the virus through possible adaptation to the FL cell strain system. At the end of the third passage in the FL cells the pooled material from the 3rd passage was subcultured into monkey kidney culture tubes to determine presence or absence of virus. The viruses which showed no demonstrable cytopathogenic effect in monkey kidney cultures were reinoculated, using the original virus, into FL cells as above. At the end of the observation period the pooled nutrient fluid was inoculated into monkey kidney cultures to determine presence of absence of virus.

It was necessary to have a control to determine if the virus could have survived in the medium for the original passage, without any multiplication occurring. Therefore, tubes containing 0.5 ml medium and no cells were inoculated with 0.1 ml per tube of the respective viruses. These tubes were incubated at 37° C for the same period of time as the FL cell culture tubes. At the end of this time 0.1 ml of

FIGURE I

SCHEMATIC PRESENTATION OF FL CELL STRAIN INOCULATION WITH THE ECHO VIRUSES



the nutrient fluid was inoculated into monkey kidney cultures (0.1 ml per tube) to determine which viruses had survived the incubation period.

The 3rd passage material from the viruses that showed cytopathogenic effect in monkey kidney cultures was inoculated into FL cell cultures. These tubes were observed and the media pooled and inoculated again into FL cultures to make a total of five passages in the FL cultures. This fifth passage material was then inoculated into monkey kidney cultures to determine presence or absence of virus.

In an attempt to attain a maximum expression of cytopathogenicity FL cell suspensions were inoculated directly with the viruses. In this situation the cell suspension was made in media containing 5% inactivated calf serum (instead of 20% human serum) and the virus was added before incubation, therefore, before the cells became established on the side of the culture tubes.

RESULTS

The data obtained from the titrations of the ECHO viruses in monkey kidney tissue cultures are presented in Table I. Also presented in Table I are comparative titrations in monkey kidney cultures obtained by other investigators. The infectivity titers (TCD_{50}), as determined by this investigation, fall in the general range of values obtained by others.

The growth of the human amnion cells, FL strain, increased approximately 2 - 3 fold in number after each transfer. The monolayer of amnion cells was not as complete as the monolayer observed with the monkey kidney cultures nor did the cells survive for as long. Cellular degeneration in uninoculated monkey kidney tissue cultures usually did not begin for approximately two to three weeks. The FL amnion cell cultures began to degenerate in ten to twelve days after a monolayer was formed.

Polioviruses types I, II, and III were inoculated into the FL strain cultures to determine if the cells were susceptible or refractive to attack by the viruses as indicated by cellular degeneration. Tubes containing FL cells were inoculated with 10^{-1} or 10^{-2} dilutions of the polioviruses. Type I caused degeneration of the cells with complete lysis in 3 days, types II and III caused degeneration of the cells beginning on the third day after inoculation; lysis was complete in 6 days. This showed that the cells were susceptible to viral attack resulting in observable cytopathogenic effects.

The results of the inoculation of the 19 prototype ECHO viruses is summarized in Table II. The first inoculation of the ECHO viruses into the FL cultures produced no observable cytopathogenic changes during

TABLE I

ECHO VIRUS INFECTIVITY TITERS (TCD_{50}) IN MONKEY KIDNEY CULTURES

ECHO Virus Type	Prototype Strain	Source of Strain	TCD_{50}/ml MSU	Stulberg[2]	NFIP[3]	Archetti[4]
1	Farouk	ATCC[1]	6.8	8.2	7.0	7.7
2	Cornelis	ATCC	6.3	7.8	6.1	5.8
3	Morrisey	ATCC	5.8	7.2	6.2	7.5
4	Pesascek	ATCC	6.5	8.5	5.8	4.5
5	Noyce	ATCC	6.5	8.5	7.7	8.8
6	D'Amori	ATCC	8.4	7.8	8.3	8.4
7	Wallace	Stulberg	8.4	7.6	8.2	8.5
8	Bryson	Stulberg	6.4	6.6	6.5	7.8
9	Hill	Stulberg	6.8	5.5	7.4	7.2
10	Lang	Stulberg	5.6	5.5	6.2	7.4
11	Gregory	Stulberg	7.1	6.7	6.9	7.0
12	Travis	Stulberg	8.2	7.5	8.3	8.8
13	Hamphill	Stulberg	7.1	7.4	6.7	8.1
14	Tow	ATCC	6.7	8.4	5.7	-
15	Charleston 96-51	Wenner	4.7	1.5	-	-
16	Harrington	Stulberg	5.5	4.5	-	-
17	CHHE-29	Wenner	5.5	-	-	-
18	Metcalf	Wenner	4.5	-	-	-
19	Burke	Wenner	7.3	-	-	-

1. American Type Culture Collection
2. Stulberg *et al.* (1958) TCD_{50}/0.1 ml
3. National Foundation for Infantile Paralysis (1957)
4. Archetti *et al.* (1957)

TABLE II

RESULTS OF INOCULATION OF ECHO VIRUSES INTO FL CULTURES AND MONKEY KIDNEY CULTURES

ECHO Virus Type	Original inoculum TCD_{50}/0.1 ml	CPE[1] in FL Cell Passage 1st	2nd	3rd	CPE in M.K.[2] after 3 passages in FL cells	CPE in M. K. after 1 passage in FL cells	CPE in M.K. after 5 passages in FL cells
1	10^6	-	-	-	-	+	
2	10^4	-	-	-	+		+
3	10^5	-	-	-	+		+
4	10^5	-	-	-	-	+	
5	10^5	-	-	-	-	+	
6	10^8	-	-	-	+		+
7	10^8	-	-	-	-	+	
8	10^4	-	-	-	-	+	
9	10^6	-	-	-	-	+	
10	10^4	-	-	-	-	+	
11	10^6	-	-	-	+		+
12	10^6	-	-	-	-	+	
13	10^5	-	-	-	-	+	
14	10^6	-	-	-	-	+	
15	10^3	-	-	-	-	-	
16	10^4	-	-	-	+		+
17	10^4	-	-	-	-	+	
18	10^3	-	-	-	-	-	
19	10^5	-	-	-	+		+

1. Cytopathogenic effect
2. Monkey kidney

(+) = cytopathogenic effect
(-) = no cytopathogenic effect

the 7 - 8 day observation period. At the end of the observation period the pooled media from each of the viruses was inoculated into another set of FL culture tubes. No cytopathogenic effect was observed for a period of 12 days. Another passage in FL cultures was made and during this third passage no cellular degeneration was caused by any of the nineteen ECHO viruses. None of the viruses in any of the three passages produced typical or observable cellular degeneration in the FL cultures.

Subculturing of the pooled, third passage material into monkey kidney cultures showed that six of the ECHO viruses caused rapid cellular destruction of the cultures. ECHO viruses types 2, 3, 6, 11, 16, and 19 caused lysis of the monkey kidney cells which began either on the first or second day after inoculation. Rapid lysis, such as this, would indicate a large amount of virus present. The remaining thirteen ECHO viruses did not cause any cytopathogenic effects in the monkey kidney cell cultures during the ten day observation period.

Since no virus was present after 3 passages in FL cultures for thirteen of the ECHO viruses it was desirable to know if any virus could be detected after a single passage in these cells. Upon reinoculation of the thirteen viruses into FL cultures and subsequent subculture of the first passage material in monkey kidney cells ECHO virus types 1, 4, 5, 7, 8, 9, 10, 12, 13, 14, and 17 caused cellular degeneration of the monkey kidney cultures. ECHO virus types 15 and 18 did not cause any cytopathogenic effects in the monkey kidney cell cultures during the twelve day observation period. Indicating that ECHO virus types 15 and 18 were destroyed during one passage in FL cell cultures.

When the ECHO viruses were incubated in medium containing no cells only ECHO virus types 7 and 14 survived the incubation period. The incubated virus material of the remaining thirteen showed no activity when tested in the susceptible monkey kidney cell cultures.

ECHO viruses 2, 3, 6, 11, 16, and 19, the viruses which caused cellular destruction of the monkey kidney cultures after three passages in FL cell cultures, were passed two more times in the amnion cell cultures. When the pooled material from the fifth passage was inoculated into monkey kidney cultures all six ECHO viruses caused cellular degeneration of the cultures which began either on the first or second day after inoculation. Because of the dilution factor involved in five passages viral multiplication must have occurred without any apparent damage to the FL cultures.

ECHO viruses 2, 3, 6, 11, 16, and 19, plus ECHO viruses types 1, 7, 9, 13, and 17, when inoculated into amnion cell suspensions did not cause cellular degeneration of the cultures. The amnion cells settled onto the wall of the tubes and formed a monolayer in the same manner as if no virus had been present. No cellular degeneration was observed in the monolayers during the ten day observation period.

DISCUSSION

The results indicate that the continuous human amnion (FL) cell line is refractive to the majority of the ECHO viruses. Thirteen of the viruses, except types 15 and 18, existed through one passage but not through three passages in the FL cell line. The presence of ECHO virus types 15 and 18 could not be detected after only one passage in FL cultures. Six of the viruses, types 2, 3, 6, 11, 16, and 19, were maintained with viral multiplication through five passages in FL cultures. None of the ECHO viruses, but all of the polioviruses, produced cellular degeneration in the cultures.

The titration values obtained in this study fall within the range of results obtained by other investigators (Table I).

The findings that the amnion cell cultures did not produce complete monolayers and that the cultures did not survive for as long as monkey kidney cultures are disadvantages in the use of the continuous amnion cell line. The less complete monolayer obtained in amnion cell cultures makes it more difficult to observe cellular degeneration. In the case of the polioviruses it did not seem to be a deterring factor because the cellular degeneration was complete, with few of the cells remaining attached to the wall of the culture tube after a period of six days. If cellular degeneration had occurred to a lesser degree visual observation of cellular degeneration would have been more difficult.

In the attempt to enhance cellular degeneration by exposure of the cell suspension to the virus there was still no observation of cytopathogenic changes, which would suggest that viral multiplication was occurring without cellular destruction.

The work of Stulberg et al. (1958) suggests that epithelial-like cell strains would be most refractive to support of ECHO virus multiplication and cytopathogenicity. They found that of five epithelial cell lines (they did not use the FL cell strain) infected with ECHO viruses none of the lines underwent cytopathogenic changes, except for an occasional ECHO type causing cellular degeneration. For the most part, the epithelial cell lines were refractive to the ECHO viruses tested. On the basis of this observation one might have suspected that the amnion cell line would also be refractive to the ECHO viruses. It does not hold true, however, that all epithelial cell lines are non-supporters of ECHO virus multiplication. The susceptible monkey kidney cultures are composed of epithelial cells and they do support viral multiplication of the ECHO viruses, with cytopathogenic changes occurring in the cultures.

The present work would indicate that the human amnion cell line is not suitable for routine ECHO virus isolation. A suitable cell line would be one in which viral activity was associated with observable cellular changes occurring in the culture. Since the FL line does not fulfill this requirement it would therefore not be suitable.

The FL line might possibly be used to separate the ECHO viruses from the polioviruses in much the same way as a selective medium might be used to separate bacterial groups, as suggested by Deinhardt and Henle (1957). Both the ECHO viruses and the polioviruses can be isolated in monkey kidney cultures from clinical specimens. However, the cytopathogenic effects of these viruses in no way distinguish them from each other. Identification is usually made through serum neutralization tests. Therefore, if it was possible to separate these groups of viruses on the basis of cell line susceptibility, identification would be faster and easier. Even though there is evidence that some of the ECHO's do multiply in the amnion cell line none show cytopathogenic effects while the polioviruses do. Before such a system could be employed, however, considerably more work would be necessary to determine the complete spectrum of viruses that cause cytopathogenic changes in FL cell cultures.

The present work does not necessarily establish that the human amnion (FL) cell line is not susceptible to cellular destruction by the ECHO viruses. There is the possibility that the FL cell line used in this laboratory is different from that of the original cell line. It is in the realm of possibility that the cells underwent modification or mutation during the time they have been carried in serial passage. It would be necessary to repeat the work in an amnion (FL) cell line maintained by another laboratory before a final conclusion could be drawn.

On the basis of amnion cell culture inoculation it would appear that the ECHO viruses could be divided into two groups: one group consisting of thirteen viruses that do not multiply nor cause cellular degeneration of the cultures and a second group of six viruses that apparently multiply without causing cellular degeneration. The significance of this grouping cannot be estimated at this time.

Lerner _et_ _al_. (1957) in investigating the susceptibility of human chorion cells to ECHO viruses types 1 - 14 found that the viruses also fell into two groups on the basis of cytopathic changes in the cultures. They found that ECHO viruses types 1, 3, 4, 6, and 11 produced cytopathogenic changes in the chorion cell cultures while types 2, 7, 8, 9, 10, 12, 13, and 14 did not. The groupings of Lerner _et_ _al_. and the groupings determined in this study, while not identical, show some overlapping. These characterizations, coupled with future investigations, might eventually lead to the recognition of divisions among the viruses of the ECHO group.

SUMMARY

The susceptibility of the continuous human amnion (FL) cell line to 19 prototype ECHO viruses was determined. None of the ECHO viruses caused cellular degeneration of FL cultures even when cell suspensions were inoculated with the viruses. Monkey kidney cell cultures were used to determine if the viruses had multiplied without causing observable cytopathogenic effects in the FL cultures.

ECHO virus types 2, 3, 6, 11, 16, and 19 caused no cytopathogenic effect through five passages in FL cultures but inoculation of monkey kidney cultures showed that viral multiplication had occurred.

After three passages in FL cultures no virus could be detected in monkey kidney cell cultures for ECHO virus types 1, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 17, and 18. Virus was detected after only one passage in FL cells for all these viruses except 15 and 18.

It was concluded that the ECHO viruses could be divided into two groups on the basis of FL cell inoculations. One group consisting of thirteen viruses that do not multiply nor cause cellular degeneration of FL cultures and a second group of six viruses that apparently multiply without causing cellular degeneration.

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SUSCEPTIBILITY OF HUMAN AMNION (FL) CELL LINE TO THE ECHO VIRUSES

By

Betsey Jane Krueger

AN ABSTRACT

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Betsey Jane Krueger

ABSTRACT

The susceptibility of the human amnion (FL) cell line to 19 strains of enteric cytopathogenic human orphan (ECHO) viruses was investigated to determine if this tissue culture strain would be suitable for routine ECHO virus isolation and to further characterize the ECHO viruses.

The cultivation of the FL cell line is described and the advantages of a continuous cell line are discussed. Culture tubes containing FL cells were inoculated with ECHO viruses types 1 - 19 and polioviruses types I, II, and III to determine if the viruses cause observable cellular degeneration (cytopathogenic changes) in the cultures.

None of the ECHO viruses, but all of the polioviruses, caused cytopathogenic changes in the cultures. Monkey kidney epithelial cell cultures were used to detect presence or absence of ECHO viruses in the nutrient medium after passage of the viruses in the FL cultures. Six of the ECHO viruses, types 2, 3, 6, 11, 16, and 19, were maintained through five passages in FL cultures. The remaining thirteen ECHO viruses, except types 15 and 18, were present after one passage but not after three passages in the FL cell line. On this basis the ECHO viruses could be divided into two groups: one group consisting of thirteen viruses that neither multiply nor cause cellular degeneration and a second group of six viruses that apparently multiply without causing observable cellular degeneration.

The study indicates that the human amnion (FL) cell line is not suitable for routine ECHO virus isolation. The possibility of using the cell line in identification of the ECHO viruses and the polioviruses is discussed.