STUDIES ON THE PROPAGATION IN VITRO OF POLIOMYELITIS VIRUSES

I. Viral Multiplication in Tissue Cultures Employing Monkey and Human Testicular Cells*

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The successful propagation of poliomyelitis virus in cellular cultures from diverse extraneural human sources (1–6) and from monkey testicular tissues (7–9) has made possible the utilization of tissue cultures for the isolation of poliomyelitis virus (3) and for the detection of specific poliomyelitis antibodies (3, 10). The discovery by Smith, Chambers, and Evans (4, 5) that human testicular cells support the growth *in vitro* of poliomyelitis viruses and the irregularities in supply of human tissues led this laboratory (7, 8) concurrently and in agreement with Dr. Evans (9) to establish successfully the ability of monkey testicular tissue to support the growth *in vitro* of the Lansing and Yale-SK strains of poliomyelitis virus. That monkey testicular cells readily support the propagation of poliomyelitis virus has been briefly recorded (7–9).[1] These preliminary studies have been amplified by further experimentation and will be presented in detail in a series of papers (11, 12). The present investigation was planned to establish unequivocal evidence for the propagation of poliomyelitis viruses in cellular cultures derived from monkey and human testicular tissues.

Material and Methods

Virus.—Five strains representative of two immunologic types of poliomyelitis virus were employed. The three strains of Type 2 virus were obtained from Dr. J. L. Melnick who kindly

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[1] While the manuscript of this paper and of the other two papers in this series were in preparation, the authors received for review the manuscripts of Youngner, Ward and Salk (*Am. J. Hyg.*, 1952, **55**, 291, 301.) and of Melnick, *et al.*, *Am. J. Hyg.*, 1952, **55**, 323, 339. The findings in these papers are not referred to in the present series of three papers since the experimental studies in the three laboratories were carried out concurrently to result in confirmatory findings in so far as similar objectives were attained. Moreover, it is plain that the principal objectives and the experimental approach employed in the present studies (11, 12) differed, with result in findings not previously reported.

supplied a Lansing strain (mouse CNS, P265) and a Yale-SK strain (Y-SK, mouse CNS, P7) and from Drs. G. C. Brown and J. D. Ainslie who provided a Lansing strain labelled AM 14-Ki-M84. The two strains of Type 1 virus were the Mahoney strain, which was obtained from Dr. Jonas Salk, and the Minnesota 1949 strain, which was isolated in this laboratory from the brain stem of a patient dead from bulbar poliomyelitis. The immediate source of virus for the present experiments was the brain and cord of monkeys or of mice that had died in from one to several passages of virus following its receipt (Lansing, Y-SK, and Mahoney strains), or isolation (Minnesota 1949). The infected tissue was triturated without abrasive to yield a 10 per cent suspension, centrifuged at 2500 R.P.M. for 10 minutes and the supernatant fluid was employed as the inoculum. The diluent used for viruses in suspension consisted of balanced salt solution, normal saline, or more commonly, 5 per cent dextrose in water.

Preservation of Virus.—Storage at the temperature of solid carbon dioxide or in 50 per cent glycerin buffered to a pH of 7.2 with phosphates was the method used for preservation of virus. The samples of virus that were employed for tissue culture studies either had been kept on dry ice,[2] or were derived directly from fresh tissue suspensions or tissue cultures. Virus in suspension was stored in sealed 2 ml. or 5 ml. ampoules.[3]

Assays for Virus.—Quantitative measurements of the Lansing and Yale-SK strains were carried out in Swiss albino mice, CFW strain, 15 to 25 days of age, by the intracranial inoculation of from 0.03 to 0.05 ml. of viral suspension. The titrations for virus utilized groups of 5 mice which had been inoculated with dilutions increasing by increments of 0.7 log save for a few initial titrations when 1 log increments were employed. Simms's 3-X6,[4] or Hanks's balanced salt solution at pH 7.0 to 7.4 was used as the diluent. Prior either to titrations or to neutralization tests, the tissue culture supernatant fluids were centrifuged at 2500 R.P.M. for 30 minutes, or at 5000 R.P.M. for 15 minutes to render them essentially cell-free. Test mice were observed daily for 28 days for the occurrence of paralysis and/or death. For the calculation of the 50 per cent end-points by the Reed and Muench method (13), the deaths that occurred during the first 2 days after inoculation were excluded.

The Mahoney strain of virus and the Minnesota 1949 strain were qualitatively assayed in macaque monkeys (*Macaca mulatta* or *Macacus cynomolgus*) by the intracranial inoculation

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[2] The capacity of poliomyelitis virus for survival in feces or nerve tissue kept at 4°C. or dry-ice temperature is established. Since similar information concerning survival of this virus in supernatant tissue culture fluid was not known to us, tests employing Type 1, Yale-SK strain, were made as follows: (1) Supernatant tissue culture fluid, which contained one part ox serum ultrafiltrate (Microbiological Associates, Bethesda, Maryland), two parts balanced salt solution (Hanks's or Simms's 3-X6), small amounts of protein from the tissue explants, (as was easily demonstrated by the addition of trichloracetic acid to the fluid) and Yale-SK virus, was divided into three aliquots, *a*, *b* and *c*. Aliquot-*a* was frozen and stored on dry ice for 4 days; aliquot-*b* was frozen and stored on dry ice for 10 days; aliquot-*c* was frozen and stored for 4 days, thawed at room temperature, immediately refrozen and stored for another 6 days on dry ice. The LD_{50} titers per 0.05 ml. in mice were (*a*) $10^{-1.6}$; (*b*) $10^{-1.7}$; (*c*) $10^{-1.4}$; as compared with a titer of $10^{-1.8}$ for the fresh unfrozen material. These variations are within the 0.4 log limits of reproducibility of mouse titrations in this laboratory. (2) In addition, virus in similar tissue culture liquids have been stored on dry ice for as long as 4 months without loss in titer. (3) Measurable disintegration of similar Yale-SK tissue culture virus when kept over a 4 hour period at room temperature in either ox serum ultrafiltrate or monkey serum chicken embryonic extract medium did not occur.

[3] Kimble Neutraglas, Toledo 1, Ohio.

[4] Microbiological Associates, Bethesda, Md.

either of 0.5 ml., or of 1.0 ml., of supernatant tissue culture fluid. Observations for signs of infection including fever were made daily for 28 days.

Preparation of Testicular Tissue for Culture.—Monkey testicular tissue was obtained commonly from fully mature *cynomolgus* monkeys, from immature *cynomolgus* monkeys, and for the first and third passages of tissue cultural series III, IV, and V, from *rhesus* monkeys. Observing surgical asepsis, the scrotum was opened by a small incision and the testicle in its tunica vaginalis was brought out through the incision. The spermatic cord and vessels were clamped with two hemostats, tied firmly with silk thread proximal to both clamps, and separated distal to the first clamp by cutting with scissors. The second clamp was employed for the transfer of the extricated testicle to a sterile container. It thereby became possible to push the stump of the cord into the inguinal canal and close with silk sutures the scrotal opening. Using sterile precautions, the tunica vaginalis was incised, and portions of the parenchyma were removed. Fresh testicular tissue was used for each new tissue passage and for each single experiment, even though it was learned that pieces of monkey testicle, measuring approximately 1 × 1 × 0.5 cm. in size, upon storage in 10 per cent monkey serum at 4°C., maintained for at least 1 week the ability to yield a "fibroblastic" outgrowth. The "fibroblastic" outgrowth was demonstrated by the transfer of explants from such tissue to roller tubes containing chicken plasma clots and a liquid medium made up of 40 or 50 per cent monkey serum and 10 or 5 per cent chicken embryonic extract.

Human testicles were obtained from elderly men who had undergone orchiectomy for its hormonal palliative effect on inoperable carcinoma of the prostate. The human testicles were provided with the tunica vaginalis intact and handled as described for the monkey testicles.

Preparation and Maintenance of Tissue Cultures.—The liquid employed in the cultivation of tissue in suspension or in plasma clots was made by the admixture of one part ox serum ultrafiltrate containing phenol red, 5 mg. per cent, and two parts of balanced salt solution (either Hanks's solution or Simms's 3-X6 solution) containing phenol red, 2 mg. per cent. The initial pH was 7.5–7.7. The sodium or potassium salt of penicillin in combination either with streptomycin, or with dihydrostreptomycin, in final concentrations of from 100 to 5000 units per ml., were included in a routine manner in the medium when modified Erlenmeyer flasks were used, but infrequently when other cultural techniques were employed. These concentrations of antibiotics were without detrimental effect either on cellular growth or viral propagation.

Chicken plasma was obtained from capons by wing bleeding utilizing a concentration of 0.2 to 0.4 mg. per cent of heparin[5] in the plasma. The plasma was stored at 4°C.

Chicken embryonic extract was prepared from 9 day embryos by the syringe method (14) modified by the use of a single stainless steel screen, 28-mesh. Extraction was performed by the addition of one volume of balanced salt solution to an equal volume of tissue pulp, followed by thorough mixing and centrifugation at 2500 R.P.M. for 20 minutes. This 1:1, or 50 per cent extract, was stored at −20°C. Prior to use, the 50 per cent extract was thawed, centrifuged at 2500 R.P.M. for 20 minutes, and only the supernatant fluid employed.

The cultivation of cells was effected by two techniques:—

(*a*) *Suspended Tissue Fragment Technique.*—Erlenmeyer flasks, 125 ml., which had been altered, as described previously (7, 8, 15, 16), by the incorporation of the side neck near the base for the insertion and withdrawal of materials, were made ready for viral inoculation by placing a circular disc of perforated cellophane of the Beckley-OC design on the bottom of the flask, by adding 5 ml. of liquid medium, and finally, by adding from 6 to 10 pieces of freshly prepared minced tissue beneath the perforated cellophane. The tissue had been minced with curved iris scissors, or two scalpel blades manipulated with scissors-like action, to result in

[5] Liquaemin, Organon, Inc., Orange, N. J.

fragments 0.5 to 1.5 mm. in maximal diameter. The flasks were assembled in groups of four in a row. The flasks were connected by short pieces of rubber and glass tubing so that 5 per cent carbon dioxide in air could be passed over the content of each flask to keep the pH at 7.2–7.6. This procedure was carried out to circumvent unavoidable gas leaks in the system which might have caused a rise in pH of the medium to 7.8–8 over a 2 to 3 day period. The fourth flask in each unit contained no tissue. The cultures were incubated at 36–37°C.

Each culture series was initiated by introducing 0.1 ml. of the supernatant fluid derived by centrifugation from a suspension of infected central nervous tissue to result in an initial dilution of virus $10^{1.7}$. At successive intervals of 3 to 4 days, the fluid overlying the cellophane was removed and replaced by 5 ml. of new medium. Even though an average of 0.7 ml. of fluid was unavoidably left beneath the cellophane, each fluid change further diluted the original virus $10^{0.9}$. Virus was transferred at intervals of 9 to 12 days to a new assembly of flasks containing fresh tissue and media to result in a further dilution of the original virus by a factor of $10^{1.7}$.

(*b*) *Combined Suspended Tissue Fragment and Plasma Clot Technique.*—Primary explants of monkey testicular tissue were placed on 11 × 22 mm. cover glasses in one drop of chicken plasma clotted with one drop of 25 per cent embryonic extract. These were inserted either into Porter flasks which were incubated in a horizontal position, or in 16 × 150 mm. test tubes, for incubation at a 5 to 10° angle from the horizontal. A liquid medium consisting of 50 per cent normal monkey serum, 10 per cent chicken embryonic extract, and 40 per cent balanced salt solution was utilized in volumes of 20 drops (approximately 1 ml.) per Porter flask and 30 drops per 16 × 150 mm. test tube. Following 3 to 5 days of incubation at 36–37°C., abundant cells were present surrounding the explants. At that time the liquid was replaced with 1 ml. of the ox serum ultrafiltrate–balanced salt solution mixture, 3 to 4 pieces of finely minced monkey testicular tissue added, and virus inoculated in a volume of 0.05 ml. The minced tissue was prepared at the time of initiation of the explant cultures. Ten to fifteen pieces were stored in 1 ml. of ox serum ultrafiltrate–salt solution mixture at 36–37°C. in 16 × 150 ml. test tubes until added to the plasma clot cultures. Subsequently the liquid was removed every 3 to 4 days and replaced with 1 ml. of ultrafiltrate–salt solution medium for Porter flasks, or 1.5 ml. for test tubes. The cells were observed while in culture and when desired the coverslips were removed for staining of the cells with Harris hematoxylin.

Control Preparations.—Controls consisted of maintaining in parallel with the test cultural units (*a*) similar units made up of virus and medium in the absence of tissue, and (*b*) source tissue cells in medium without virus. The first control established the duration of survival of the virus that was derived from the initial inoculum, and the second control served to rule out the presence in the tissue of extraneous virus derived from the host and cytopathogenic material other than poliomyelitis virus. These two sets of controls showed (*a*) that strains of poliomyelitis virus, Type 2, persisted at 37°C. for 3 to 4 days in the absence of tissue, but were never recoverable at the end of the first tissue passage (9 to 11 days); (*b*) that extraneous virus pathogenic for mice or monkeys, or non-specific cellular toxins were not encountered in these studies.

Bacteriological Studies.—Liquid thioglycollate medium was employed as a routine for the detection of extraneous contaminants (*a*) in suspensions of central nervous tissue, (*b*) in tissue culture fluid containing virus, and (*c*) in the ingredients employed for tissue culture.

RESULTS

Eight series of experiments (series I–VIII) were carried out to establish unequivocal evidence for the propagation *in vitro* of poliomyelitis virus. It was found, early in these studies for three series (8), that monkey and human testicular cells support propagation *in vitro* of murine adapted strains of

Type 2. The present studies were carried out in continuation and extension of these earlier findings.

Cultures in Monkey Testicular Cells

Poliomyelitis Virus, Type 1 (Brunhilde Type).—The successful propagation of poliomyelitis virus, Type 2, Lansing and Y-SK strains in monkey testicular

TABLE I

Propagation in Vitro of Poliomyelitis Virus, Type 1 (Brunhilde) in Cultures of Monkey Testicular Tissue

No. of tissue passage	Total time in culture		Cumulative log of dilution based on:—				Monkey infectivity of tissue culture fluid	
			Tissue change		Fluid change			
	Mah.*	Minn.*	Mah.	Minn.	Mah.	Minn.	Mah.	Minn.
	days	*days*						
1	11		3.7		4.6		Yes‡	
		11		2.7		4.5		N.T.‡
2	21		5.4		9.0		Yes	
		20		4.4		8.0		Yes
	32		7.1		11.6		Yes	
3	35				12.5		Yes	
		31		6.1		11.5		N.T.
4	43		8.8		16.0		Yes	
		42		7.8		15.0		Yes
5	52		10.5		19.5		Yes	
7		68		11.7		26.7		Yes
8	85		15.6		30.0		Yes	N.T.

* Mah.—series I, Mahoney strain, Type 1. Minn.—series II, Minnesota 1949 strain, Type 1.

‡ Monkeys were observed for a 28 day period for the occurrence of fever and paralytic symptoms. "Yes" indicates the occurrence of paralysis and/or death, plus the presence of histological evidence of poliomyelitis infection in the central nervous system; "N.T." signifies not tested.

cells (7, 8) led to attempts to apply the same techniques for the cultivation of two strains of Brunhilde type by carrying out two series of tissue cultures, series I and series II. The data that relate to these two series are presented in Table I.

It can be seen from series I that propagation of the Mahoney strain was carried out successfully over a period of 85 days by employing the suspended tissue fragment technique. The inoculum was a 1 per cent suspension of brain

and cord tissue. Each fluid change at an interval of 3 to 4 days resulted in a log dilution of 0.9 and each successive tissue replacement at intervals of 9 to 11 days in a log dilution of 1.7. The resultant cumulative dilution of brain tissue as calculated from eight tissue changes was $10^{15.6}$, or from 24 fluid changes, 10^{30}. The presence of virus in the original inoculum and in aliquots from the first, second, third, fourth, fifth and eighth tissue passage fluids was established by the production in monkeys of paralysis and/or death and the

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Evidence for the Propagation of Poliomyelitis Virus

Series No.	Tissue passage No.		1	2	3	4	5	6	7	8	9
	Cumulative time in culture, *days*		9	19	28	37	46	55	64	73	82
III	Cumulative log of dilution based on	Tissue change	2.7	4.4	6.1	7.8	9.5	11.2	12.9	14.6	16.3
		Fluid change	4.5	8.0	11.5	15.0	18.5	22.0	25.5	29.0	32.5
	Test for virus in culture liquid on passage days	6–7	5/6*	7/8	5/6	6/8	5/6	6/7	6/6	5/6	6/6
		9–11	N.T.	6/6	6/7	5/7	6/6	5/6	5/6	6/6	5/6
	Cumulative time in culture, *days*		12	21	31	40	49	58	67	76	85
IV	Cumulative log of dilution based on	Tissue change	2.7	4.4	6.1	7.8	9.5	11.2	12.9	14.6	16.3
		Fluid change	5.4	8.9	12.4	15.9	19.4	22.0	25.5	29.0	32.5
	Test for virus in culture liquid on passage days	6–7	5/7	5/5	7/9	5/6	5/6	6/6	2/6	5/6	5/6
		9–11	3/7	N.T.	N.T.	5/6	5/6		5/6	6/7	4/6

* Denominator signifies the number of mice inoculated; numerator, the number dead from poliomyeliti

histological evidence of poliomyelitis. These findings established conclusively the propagation *in vitro* of the Mahoney strain of poliomyelitis virus.

In confirmation of the findings that had been obtained with the Mahoney strain, the Minnesota 1949 strain of Type 1 was similarly employed in series II for cultural studies. Moreover, the utilization of the Minnesota 1949 strain made it possible to learn whether a newly isolated strain would readily adapt itself to propagation in monkey testicular cells cultivated *in vitro*. The data that relate to the propagation of the Minnesota 1949 strain are included in Table I. Suspended tissue fragment cultures were employed for the first four

tissue passages, and the combined suspended tissue fragment and plasma clot cultural technique for passages five through seven. The latter technique resulted in a dilution of $10^{1.3}$ for each tissue or fluid replacement.

It can be seen that the dilution of the virus contained in the original tissue after seven transfers to new flasks and replacement of the overlying fluid was $10^{11.7}$, as estimated by tissue replacement, or $10^{26.7}$, as measured by fluid replacement. Since our limited supply of monkeys made it impossible to deter-

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e 2, Shown by the Dilution of the Original Inoculum

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
100	111	122	132	143	153	164	175	185	196	207	218	228	238	245	256	263
19.7	21.4	23.1	24.8	26.5	28.2	29.9	31.6	33.3	35.0	36.7	38.0	39.3	40.6	41.9	43.2	44.5
39.5	43.0	46.5	50.0	53.5	57.0	60.5	64.0	67.5	71.0	74.5	78.4	82.3	86.2	88.8	92.7	95.3
5/5	6/6	4/4	3/3	3/5	5/5	5/5	5/5	4/4	N.T.	3/3	N.T.	3/4	5/5	1/4	7/7	5/5
5/6	4/5	3/3	4/4	0/5	5/5	2/5	4/5	N.T.	4/4	4/4	6/6	N.T.	6/6	N.T.	5/5	4/4
103	112	123	133	144	154	165	176	186	196	207	217					
19.7	21.4	23.1	24.8	26.5	28.2	29.9	31.6	33.3	35.0	36.7	38.4					
39.5	43.0	46.5	50.0	53.5	57.0	60.5	64.0	67.5	71.0	74.5	78.0					
4/4	5/5	5/5	5/5	3/4	1/5	5/5	4/4	4/4	N.T.	4/4	4/4					
3/3	4/4	5/5	5/5	2/5	5/5	5/5	5/5	N.T.	4/4	3/4	4/4					

mine quantitatively the infectious titer either of the original inoculum or of the end-product, qualitative tests were made to learn whether sufficient virus was present to paralyze and/or kill the recipient monkeys. It can be seen that the supernatant tissue fluids from passages two, four, and seven contained poliomyelitis virus.

The results of series II confirmed the findings of series I by showing that the freshly isolated Minnesota 1949 strain, of Type 1 poliomyelitis virus, propagated in cultures of monkey testicular tissue as readily as the Mahoney strain.

Poliomyelitis Virus, Type 2 (Lansing Type).—Four series of tissue cultures were initiated by utilizing as the inoculum strains belonging to Type 2. The results of series III and series IV are presented jointly in Table II, since the Y-SK strain was employed for both series. These two series were maintained in parallel by transfer to fresh monkey testicular cells at intervals of 9 to 11 days for 21 passages. Series III was maintained for 21 passages by utilizing the suspended tissue fragment technique and, to date, for 6 additional passages by employing combined suspended tissue fragment and plasma clot cultures. Fluid representative of each tissue passage was tested qualitatively in mice for the presence of virus. Series IV was carried through 22 tissue passages by employing the suspended tissue fragment method of culture.

TABLE III

Additional Evidence for the Propagation of Poliomyelitis Virus, Type 2, Yale-SK Strain in Two Series of Monkey Testicular Tissue Cultures

No. of tissue passage	Mouse titration, Negative Log of LD_{50}/0.05 ml.		Monkey infectivity		Neutralization tests	
	Series III	Series IV	Series III	Series IV	Series III	Series IV
Inoculum	3.1	3.1				
6			Yes*			
7	3.0			Yes	Yes‡	
8		2.4				
10	1.8					
12	1.7					
14	2.3		Yes			
20			Yes			
21	1.7		Yes (?)§		Yes	
22		2.3		Yes		Yes
23			Yes			
27		Discontinued	Yes			

* The occurrence of paralysis and/or death of one monkey within 28 days following intracranial inoculation and confirmatory histopathological evidence of poliomyelitis infection in the central nervous system.

‡ The occurrence of complete neutralization of virus with undiluted serum, as demonstrated by inoculation intracerebrally into mice.

§ Three monkeys were employed to test fluid from this passage. Two developed fever on the 7th to 9th day after inoculation; none showed evidence of paralysis. All three monkeys were subsequently immune to intracerebral challenge with Yale-SK virus.

It can be seen from series III that virus was propagated over a period of 263 days for 27 passages to result in a dilution of original central nervous system tissue of $10^{44.5}$ by tissue replacements, or $10^{95.3}$ by fluid replacements. Series IV was carried for 217 days by which time the original virus had been diluted $10^{38.4}$ by tissue replacement or $10^{78.0}$ by fluid replacement.

Additional evidence in support of propagation of the Yale-SK strain in the two series of cultures is presented summarily in Table III. This evidence consisted of testing the supernatant fluid (*a*) by quantitative titration for its capacity to produce paralysis and death of mice, (*b*) by assessing its ability to produce paralysis and/or death and histological evidence of poliomyelitis in monkeys, and finally, (*c*) by the neutralization technique in mice utilizing specific type antiserum obtained from Dr. J. E. Salk for identification of the virus as Type 2. It can be seen (*a*) that the LD_{50} for mice varied from $10^{1.7}$ to $10^{3.0}$ throughout the studies, (*b*) that the virus retained its ability to produce poliomyelitis in monkeys, and finally, (*c*) that the virus contained in the supernatant fluid of tissue passage 7 and 21 of series III and 22 of series IV was established as being poliomyelitis virus, Type 2.

From the data summarized in Tables II and III, it may be concluded that unequivocal evidence has been established for the propagation of poliomyelitis virus, Yale-SK strain, in monkey testicular tissue cultures. This evidence of the adaptation of poliomyelitis virus to cells derived from an extraneural source was not accompanied by any striking change in the propensity of the virus to multiply in these cells. Moreover, the expected findings of retention of antigenicity was realized, but an anticipated alteration in the pathogenicity of this strain of virus has not yet been established convincingly in either of these two series of tissue cultures.

Lansing virus (series V) was maintained successfully through eight tissue passages, as reported previously (7, 8). In an attempt at continuation of this passage series, numerous efforts to carry out subsequent passages from the eighth passage fluid failed to propagate an agent pathogenic for mouse or monkey. Similarly, an attempt to repeat the eighth passage using stored seventh passage fluid as inoculum also failed. It was concluded that the virus in this seventh passage fluid was in an amount insufficient to initiate viral multiplication within the cells.

Because of these results a second Lansing series (series VI) was started with a strain of Lansing virus from the University of Michigan. An identical technique of suspended tissue fragment cultures in modified Erlenmeyer flasks was utilized. Three tissue passages over a period of 34 days were carried out. Virus definitely propagated during this period. However, the series was discontinued because the viral concentrations being maintained were low. These findings were accepted by us as evidence to suggest strongly that the Lansing strain of poliomyelitis virus, Type 2, grows less well in monkey testicular cultures than the Yale-SK strain. Conversely, the ease with which the Yale-SK strain was adapted to monkey testicular cells and maintained by culture *in vitro* led to the use of the Yale-SK virus for the studies that were concerned with growth curves and cytopathogenic effects (11, 12).

Cultures in Human Testicular Cells

When human testicular cellular cultures were found by Smith *et al.* (4, 5) to support the growth of the Lansing and Hof. strains of poliomyelitis virus, two series of experiments, series VII and series VIII, were carried out to confirm their findings.

TABLE IV

Growth of Poliomyelitis Viruses in Cultures of Human Testicular Tissue

No. of tissue passages	Cumulative log of dilution based on		Frequency of death in mice				Negative log of* LD_{50}/0.05 ml.	
			Series VII Lansing		Series VIII Yale-SK			
	Tissue change	Fluid change	Fluid from days of cultivation				Lansing	Yale-SK
			6 or 7	9–11	6 or 7	9–11		
Inoculum							4.1	3.9
1 (Control)	2.7	4.5		(0/4)§		(0/3)		
2 (Control)	4.4	8.0		2/3		2/2		
3 (Control)	6.1	11.5	2/3	3/4 (0/4)	3/3	4/4 (0/3)		2.0
4 (Control)	7.8	15.0	2/4	0/4 (0/4)	3/3	9/9 (0/4)		2.5

‡ (Control) indicates that a flask containing virus and liquid media but no tissue was handled in a manner similar to the cultural flasks.

* The method of Reed and Muench was used to determine the LD_{50} value. Mice were observed daily for 28 days after the intracranial inoculation of 0.03 to 0.05 ml. of inoculum.

§ The numerator indicates the number of mice dying during the period 3 to 28 days after inoculation. The denominator signifies the number of mice injected.

Poliomyelitis Virus, Type 2.—The technique of suspended tissue fragment cultures in modified Erlenmeyer flasks was employed. The inoculum consisted of 0.1 ml. of a suspension of the Lansing strain (series VII) with an LD_{50} of 4.1 in 0.05 ml. and of 0.1 ml. of a suspension of the Yale-SK strain (series VIII) with an LD_{50} of 3.9 in 0.05 ml. Four tissue passages were effected in each passage series. The tissue replacements were made at intervals of from 9 to 11 days to result in successive dilutions of 1.7 log; the fluid was changed at intervals of 3 to 4 days to result in successive dilutions of 0.9 log. The results of the two passage series are presented in Table IV.

It can be seen from Table IV for series VII and for series VIII that virus persisted in the tissue but not in the control flasks for four tissue passages. The resultant cumulative log of the dilution for tissue changes, 7.8 and for fluid changes, 15.0, when compared with the LD_{50} of the inoculum for each series, make apparent the extent of multiplication, and were accepted as confirmatory of the earlier findings (4, 5). Both series were discontinued and source fluid from the final passage was placed in storage for future use.

DISCUSSION

The present observations establish the fact that poliomyelitis virus can propagate readily in extraneural tissue. The evidence is found in the repeated demonstration that monkey or human testicular cells kept under cultivation in the suspended tissue fragment type of culture supported the growth *in vitro* of five strains representative of two immunologic types of poliomyelitis virus. For example, for one passage series, poliomyelitis virus, Type 2, Y-SK strain, had been maintained for 263 days to yield a minimal dilution factor calculated by tissue replacements of $10^{44.5}$ or assessed by fluid replacements of $10^{95.3}$. Moreover, the three criteria of Robbins and Enders (17) for successful viral cultivation were satisfied for each of the five strains of poliomyelitis virus in tissue passage series: (*a*) the dilution factors of the supernatant fluids to date ranged from 10^{15} to $10^{95.3}$, thereby being vastly in excess of the titer of $10^{3.1}$ of the original viral inoculum; (*b*) single cultures were demonstrated repeatedly to contain more virus than was represented by the inoculum (11); (*c*) plasma clot cultures tested for each virus employed revealed cytopathogenic effects (11, 12) in from 6 to 10 days after inoculation. Finally, the identity of each of the five strains was established by neutralization tests and by the histopathological findings in monkeys dead from the injection of tissue culture virus. The foregoing composite observations, together with the unequivocal evidence for propagation *in vitro* of virus when a line of fibroblastic cells derived from monkey testicular tissue was employed (12) make it plain that extraneural cells free from neural elements can provide adequately for the growth of poliomyelitis virus.

A wide variety of cells when maintained successfully in tissue culture are known to satisfy the growth requirements of poliomyelitis virus. Human embryonic brain and cord were employed for the cultivation of the MV (18), Lansing (1, 6), and Brunhilde (2) strains; a mixture of human embryonic skin, muscle, and connective tissue for the Lansing (1, 6) and Brunhilde (2) strains; human foreskin and subcutaneous tissues from patients ranging in age from 4 to 11 years for the Lansing strain (2); human embryonic intestine for the Lansing strain (1, 6); human adult testicular tissue for the Lansing and Hof. strains (4, 5); and monkey testicular tissue for each of two immunologic types of poliomyelitis virus (7–9). In this laboratory, and others, the attempts

to establish a source of human tissues for *in vitro* cultural studies have met commonly with difficulties in procurement when a given type of tissue must be available on a given day. This problem was simplified by the discovery that monkey testicular cells *in vitro* satisfy the growth requirements of poliomyelitis virus since male monkeys are at hand in most laboratories engaged in poliomyelitis studies. Indeed, the maintenance of susceptible monkey testicular cells in tissue culture provides a readily applicable and highly efficacious method for the production of poliomyelitis virus under conditions that are simple and subject to accurate control. Moreover, the use of these cells in other tissue culture techniques has made possible studies to determine the factors that influence the growth requirements of poliomyelitis virus and may make possible the production in quantity of virus for prophylactic purposes, diagnostic tests, and chemotherapeutic studies.

SUMMARY

Poliomyelitis virus was propagated *in vitro* successfully in extraneural tissues. Suspended tissue fragment cultures and combined plasma clot–suspended tissue fragment cultures of monkey or human testicular tissues were employed. Five strains representative of poliomyelitis virus were maintained for from 36 to 263 days in the suspended tissue fragment type of culture. The dilution factors calculated by tissue replacements for the eight serial passages ranged from $10^{7.8}$ to $10^{44.5}$ and when assessed by fluid replacements, from 10^{15} to $10^{95.3}$. The LD_{50} for each strain of Type 2 virus was determined for selected transfers. The identify of each strain of virus was established by neutralization tests and histopathological findings in monkeys dead from the injection of tissue culture virus. Control experiments and other tests made known that propagation of poliomyelitis virus did not occur in the absence of viable testicular cells and that an extraneous virus was not inadvertently acquired during the course of these studies.

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