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Multiplication of Virulent Poliovirus in Capuchin Monkey Kidney Cultures Without Microscopically Observed Cytopathogenicity.* (22098)

ALBERT S. KAPLAN† AND JOSEPH L. MELNICK.
Section of Preventive Medicine, Yale University School of Medicine, New Haven.

Previous work from this laboratory demonstrated that the South American ringtail monkey (*Cebus capucina*) has a limited susceptibility to poliovirus *in vivo*(1). Recent experiments showed that the Type 2 Y-SK strain, highly cytopathic for rhesus kidney cultures, was without demonstrable effect in capuchin cultures(2). The present report extends the earlier findings in demonstrating that Type 1 human strains can multiply in epithelial cultures of capuchin kidney, even though no obvious cytopathic changes could be observed. In the course of these experiments, material became available to determine whether capuchin cultures, which supported virus growth with no apparent cell destruction, could act as a selective medium for the propagation of non-neurotropic variants of poliovirus‡(3-8).

Materials and methods. Four Type 1, four Type 2 and three Type 3 strains were employed, both as stool suspensions of patients ill with poliomyelitis and as the first passage material from infected rhesus kidney cultures (in which they were all markedly cytopathogenic). The procedures in use in this laboratory for growing cultures, for isolation and titration of viruses have been reviewed recently(9). In order to test for survival of virus under the conditions of the experiments, control tubes were inoculated and incubated together with the capuchin cultures. The control tubes contained medium alone (without cells) or rhesus cells that had been killed by freezing. Normal controls of uninoculated capuchin cultures were also included. For each test 0.1 ml of fluid was inoculated into a series of 8 to 10 monolayer cultures of capuchin kidney cells and 0.9 ml of lactalbumin hydrolysate medium(9) was added. The cultures were allowed to incubate for various times; at the end of each incubation period, the fluids were harvested, and fresh medium was added if the incubation was to be continued. Titrations were carried out in rhesus kidney cultures, using 8 tubes per dilution, since the capuchin cultures showed no apparent cytopathic alterations. The plaque technic was carried out according to the method of Dulbecco and Vogt(10). Tests for neurotropic properties of the Type 1 strains were carried out by inoculating the first or second passage capuchin culture fluid into

* Aided by grant from the National Foundation for Infantile Paralysis.

† Present address: Department of Bacteriology, University of Illinois, Urbana.

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TABLE I. Passage of Type 1 Poliovirus in Cultures of Capuchin Monkey Kidney.

Strain	Passage in capuchin cultures	Conc. of TC fluid inoc.	Incubation period (days)	TCD_{50} titer in rhesus cultures (per ml)
Easton -15	1	10^{0}	14	5.0*
	2	"	14	4.1*
	3	10^{-1}	7	>5.0
		10^{-2}	7	4.6
		10^{-3}	7	2.0
		"	11	>4.0
	4	10^{-3}†	7	1.7
		" †	11	3.1
		" †	18	4.0
	5	10^{0}	2	4.6
NE-23	1	10^{0}	14	4.3*
	2	10^{-1}	7	5.8
		10^{-3}	7	>4.0
		"	11	>5.0
	3	10^{-4}†	7	3.0
		" †	11	4.3
		" †	18	4.4
	4	10^{0}	2	4.7
NE-34	1	10^{0}	14	4.0*
	2	10^{-1}	7	5.4
		10^{-3}	7	>4.0
		"	11	>5.0
	3	10^{-4}†	7	2.8
		" †	11	2.8
		" †	18	3.6
	4	10^{0}	2	5.1

* Virus could not be recovered from tubes containing medium alone or rhesus cells killed by freezing, which were inoculated with the same amount of virus and incubated for the same period of time as these capuchin cultures.

† These dilutions were made of the fluids harvested from the cultures of the previous passage which had been inoculated with virus diluted a thousandfold.

rhesus monkeys, 2 intracerebrally (0.5 ml) and 2 intraspinally (0.1 ml). The 4th or 5th passage material was tested in 6 monkeys for each of the strains.

Results. The Type 1 strains grew readily in trypsinized capuchin kidney cultures, although no cytopathic changes could be observed in the microscope. The infected cultures looked exactly like the uninoculated controls. No evidence could be obtained to show that Type 2 and 3 strains multiplied under the same circumstances. The results of the titrations and the number of passages for three Type 1 strains are given in Table I. There are 3 lines of evidence to show that multiplication of virus occurred:

1. All the passages were made by the addition of 0.1 ml of virus or harvested fluids into 0.9 ml of medium in the culture tubes. Successive dilutions for the 5 passages of strain E-15 were 10^{1}, 10^{1}, 10^{4}, 10^{4}, and 10^{1}, making the final dilution of the original material 10^{11}. The titer of virus was never greater than 10^{5} TCD_{50}/ml, and since virus was present in the final fluid at a titer of $10^{4.6}$, virus multiplication must have occurred. This is supported by the failure to obtain virus from the control tubes (no living cells present) incubated during the first two passages together with the capuchin cultures.

Similar considerations apply to strains NE-23 and NE-34; these also showed the presence of virus at relatively high levels in the final culture fluids, which represented a dilution of 10^{11} of the original fluids.

2. Multiplication was shown as well by the increase of virus during a single passage. When capuchin cultures were inoculated with less than 10 TCD_{50} of strain E-15 in its second passage, fluids with a titer of greater than $10^{4.0}$ were obtained, indicating that at least a thousandfold increase in virus concentration had occurred. This type of virus increase was obtained several times and also with the other Type 1 strains.

3. Virus propagation was also demonstrated by titrations of fluids taken at different times from the culture tubes. With passage 3 of the E-15 strain, fluids harvested from the cultures inoculated with 10 TCD_{50} yielded a titer of $10^{2.0}$ at the end of 7 days and one greater than $10^{4.0}$ at the end of 11 days of incubation. This increase of virus concentration with time is shown also in passage 4: the titers of the harvested fluids were $10^{1.7}$, $10^{3.1}$, and $10^{4.0}$ at the end of 7, 11, and 18 days, respectively. Similar results were obtained with the other two strains.

To determine whether all the capuchin cells in the culture produced virus but without cellular degeneration or whether only a few cells were involved in virus propagation, the following experiment was done.§ Capuchin monkey kidney cells were grown in petri

§ These experiments were carried out by one of us (A.S.K.) at the California Institute of Technology through the hospitality of Drs. Renato Dulbecco and Marguerite Vogt.

TABLE II. Number of Infected Cells in Capuchin Monkey Kidney Cultures as Determined by the Plaque Technic.

Day	No. of cells in inoculum (× 100,000)	No. of plaques	% of cells infected
1	2	105	.05
2	1.1	31	.03
3	1.8	27	.015
6	.1	21	.21
7	1.7	39	.023

plates according to the technic of Dulbecco and Vogt(10). These monolayer cultures were inoculated with 0.1 ml of Easton-15, 4th passage capuchin virus; the inoculum contained 2 x 10^5 plaque-forming particles (as determined by titration on rhesus plates). Nutrient medium was added, and at the intervals shown in Table II, the medium was removed, the plates washed several times with buffered saline, and the monolayer of cells suspended by means of 0.02% versene. The cells were sedimented and resuspended in 1 ml of nutrient medium; 0.1 ml of this cell suspension was seeded onto petri plate cultures of rhesus monkey kidney cells. After a period of 30 minutes to allow for adsorption of virus, an agar overlay was added. The plaques were counted at the end of 4 days.

The results shown in Table II demonstrate that in a population of 10^5 cells only an average of 60 cells was infected. This experiment shows that the absence of obvious cytopathic changes in the capuchin monkey kidney cultures was due to the fact that only a small proportion of cells was involved in the production of virus. The degeneration of so few cells in the culture would be very difficult to detect microscopically. The propagation of certain polioviruses in tissue cultures in the absence of observed cytopathic changes has previously been recorded(12-14). and it is likely that the present explanation of only a small proportion of cells supporting virus propagation, underlies the earlier observations.

Neurotropic properties. After 4 to 5 passages in capuchin cultures the 3 Type 1 strains were compared for the ability to grow in the central nervous system of rhesus monkeys. Attenuated strains of poliovirus have been characterized by their restricted growth in the monkey nervous system. limited chiefly to the lumbar part of the spinal cord(11). Thus an intracerebral inoculation of attenuated virus would be expected to produce disease rarely—only if the virus in some manner found its way to the lumbar region. As a corollary, intraspinal inoculation would be expected to produce paralysis of the legs, if at all. In the present experiments the viruses

TABLE III. Neurotropism of Poliovirus in Rhesus Monkeys, after Propagation without Microscopically Observed Cytopathogenicity in Capuchin Cultures.

Strain	No. of passages in TC	Route	Doses* (× 1000)	Result,† day of paralysis
E-15	2	Intracerebral	8	7 (L); inc.
		Intraspinal	1.6	4 (L→A); remained well
	5	Intracerebral	20	7 (A + L); 10 (L); 14 (A + L)
		Intraspinal	4	4 (L→A); 4 (L→A); 4 (L)
NE-23	1	Intracerebral	5	8 (A + L); 8 (A)
		Intraspinal	1	4 (L); 4 (L)
	4	Intracerebral	25	14 (T + L); 14 (T + L); inc.
		Intraspinal	5	2 (L→A); 3 (L→A); 3 (L→A)
NE-34	1	Intracerebral	0.8	5 (L); inc.
		Intraspinal	0.2	4 (L); inc.
	4	Intracerebral	63	8 (A + L); 11 (L); 14 (L)
		Intraspinal	12.6	3 (L→A); 4 (L→A); 4(L)

* Doses in inoculum determined as TCD_{50} for rhesus kidney cultures on the day monkeys were inoculated.

† L = leg paralysis; A = arm paralysis; T = tremors; inc. = incomplete test due to nonspecific death of monkey. 4 (L→A) indicates that paralysis first occurred on the 4th day, the legs being involved. Subsequently the arms (A) became involved.

propagated in capuchin kidney cells remained virulent, in the doses inoculated, after 4 or 5 serial tissue culture passages. As shown in Table III, paralytic disease was produced after intracerebral inoculation. In addition, the affinity of the virus for the CNS was demonstrated after intraspinal inoculation. When the virus was injected into the lumbar area, it multiplied there and frequently ascended the cord; this manifested itself by leg paralysis followed by arm paralysis.

Summary. Cultures of trypsinized kidneys of South American capuchin monkeys (*Cebus capucina*) supported the multiplication of Type 1 polioviruses, even though no cytopathic changes were observed by microscopic examination of the cultures. The failure to observe cytologic changes was found to be due to the small proportion of cells in the culture capable of supporting the growth of virus. After 4 to 5 tissue culture passages the viruses remained virulent for the monkey brain and spinal cord. No evidence was obtained for the propagation of Type 2 and 3 strains in the capuchin cultures.

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Measure of Irritation by Quantitative Determination of Extravasated Dye.* (22099)

R. A. SACHS AND W. L. LUMMIS. (Introduced by M. H. Kuizenga.)
Department of Biological Control, The Upjohn Co., Kalamazoo, Mich.

Intravenously administered colloidal dyes have been observed to concentrate at sites of inflammation(1-5). This phenomenon has been the basis for tests to measure the intensity of irritation(6-10). In such tests, visual estimations of the quantity of dye at irritated sites were graded by various systems, either direct or by comparison to standard color charts. The results of visual observations in this manner are influenced by subjective bias and are not easily reproducible.

This report describes a method for the quantitative removal of accumulated Evans blue dye from irritated tissue and its measurement spectrophotometrically. This should be a reliable index of the degree of irritation, provided the amount of extravasated dye is proportional to the extent of irritation. A modification of the method of Caster, Simon, and Armstrong(11,12) for the measurement of plasma volume was used for extracting the dye from tissue.

Methods and materials. White rats, (Sprague-Dawley), 115-130 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital. The skin on the posterior side of the hind legs was slit to expose the gastrocnemius muscles and 0.03 cc of the test material was injected into each muscle using a ¼ inch, 27 gauge needle. Fifteen minutes after the injection of the test material,

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