Cultivation of Poliomyelitis Virus in Tissue Culture III. Synthetic Medium in Roller Tube Cultures.* (19901)

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(Introduced by R. C. Parker.)

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We have reported(1-4) that a synthetic nutrient medium, Mixture No. 199, devised by Morgan, Morton, and Parker(5,6) can be used in the cultivation of Lansing poliomyelitis virus. In these studies, the virus was cultivated in Maitland-type (Erlenmeyer flask) cultures of tissue derived from human embryonic brain and spinal cord, human embryonic kidney, tonsils of children, and rhesus monkey testis, kidney, lung, and other organs. It is the purpose of the present paper to report that several strains of the Brunhilde, Leon, and Lansing types can be grown in roller tube cultures of tissue from monkey testis. In these cultures, Mixture 199 has been the only added source of nutriment.

Materials and methods. Preparation, maintenance, and infection of cultures. Methods were based on those introduced by Enders and his associates for the cultivation of vaccinia (7) and poliomyelitis(8) viruses, and later modified by others(9,10). In our experiments, however, Mixture 199 was used as nutrient in place of the more complete feeding mixtures containing such naturally-occurring ingredients as embryo extract, horse serum and serum ultrafiltrate, used by other workers. Testes of immature rhesus monkeys were minced finely in Mixture 199 containing penicillin (100 units/ml) and streptomycin (100 μg/ml). Two drops of reconstituted chicken plasma (Difco) were added to rimless test tubes (15 $\times$ 1.5 cm) and spread evenly over the lower third. Eight to 12 fragments of testicular tissue were then placed on the plasma-lined surface. Clotting was accomplished by the addition of one drop of reconstituted chick embryo extract (Difco). A volume of 2 ml of Mixture 199 was then added to the cultures. Some cultures were also prepared in larger tubes (23 $\times$ 5 cm) to which were added 15 drops of plasma, about 100 fragments of tissue, 5 drops of extract, and 20 ml of Mixture 199. All cultures were rotated mechanically at 37°C. The tissue fragments were examined microscopically each day, and as soon as an abundant outgrowth of fibroblasts was observed, the cultures were infected with virus. The time required for adequate outgrowth of fibroblasts was usually about 7 days; during this period, no fluid changes had to be made because of the buffering action of Mixture 199. The outgrowth of fibroblasts was somewhat less abundant than we have observed in cultures treated with mixtures containing naturally-occurring ingredients. Cultures in the smaller tubes were infected by removing all nutrient and replacing with 2 ml of Mixture 199 to which an appropriate amount of virus-infected fluid had been added; in the larger tubes, the volume was 20 ml. In the beginning, cultures of virus were initiated by adding infected material in the form of mouse CNS (Lansing strain), monkey CNS (Brunhilde, Canadian Eskimo, Y-SK, and Leon strains), or infected tissue culture fluid (Mahoney, MEF1, and Saukett). Subcultivation was continued by inoculating infected culture fluids, removed 16-30 days after initiation of the culture, into 7-day-old cultures of monkey testis. The outgrowths of fibroblasts were examined daily for cytopathogenic changes(8-10). If the object of the experiment was to harvest the maximal volume of virus-infected fluid, changes of nutrient were made every 4 days. If the experiment was one in which virus or serum was being titrated, the fluids were not changed, and the tests were read 6-8 days after the addition of virus or virus-serum mixtures to the cultures. *Titration of viruses and antisera.* Culture fluids infected with some of the strains mentioned were titrated by the inocu-

* Aided by a Grant from The National Foundation for Infantile Paralysis.

lation of 0.2 ml of 10-fold dilutions into 2 or 3 cultures of monkey testicular tissue showing adequate outgrowth of fibroblasts. The titer of a virus suspension was the highest dilution which produced cytopathogenic (degenerative) changes in all of the inoculated cultures within 6-8 days, the fluids not being changed during this period. For convenience, this titer may be designated the CPD (cytopathogenic dose). A refinement of technic would be the inoculation of 8-10 tubes, with the calculation of the CPD_{50}. Poliomyelitis antisera prepared in monkeys in our laboratory were also titrated. Suitable 10-fold dilutions of serum were mixed with sufficient virus-infected tissue culture fluid to give a final amount of virus of 50 CPD. The mixtures were held at room temperature for 1½ hours, then inoculated into 2 or 3 cultures of monkey testis showing adequate outgrowth of fibroblasts. A titration of the virus-infected fluid added to the serum mixtures was performed with each batch of serum tests. The neutralizing endpoint of the antiserum was determined by the highest dilution which completely inhibited the cytopathogenic action of the added virus in 6-8 days, the fluid not being changed during this period. This neutralizing titer may be designated the CPID (cytopathogenic inhibiting dose). Cytopathogenic inhibiting titers are expressed as dilutions of serum before the addition of an equal part of virus, and are not therefore "final" titers. *Immunization of monkeys.* Groups of rhesus monkeys were immunized in order to examine the antigenicity of Lansing and Brunhilde viruses cultivated in the presence of Mixture 199. Four monkeys received every 2 weeks, by the intramuscular route, Lansing virus grown in cultures of monkey testicular tissue in large roller tubes. Six monkeys were likewise injected with tissue-cultivated Brunhilde virus. A third group was inoculated with Brunhilde virus in the form of a pool of infected monkey CNS tissue. The pool used was prepared from the cord enlargements of monkeys harvested on the first day of paralysis following the thalamic inoculation of 0.8 ml of a 1 in 10 suspension of a Brunhilde seed pool. The pool was not titrated in monkeys, but another pool of the same virus prepared by similar methods at about the same time had a PD_{50} titer in monkeys of $10^{-5.2}$ (0.8 ml volumes thalamically). All 3 groups received the virus inoculum incorporated in an equal volume of Freund's adjuvant (without mycobacteria), since it has been found that the use of adjuvant leads to an increase in antigenicity(11-13). The animals were bled at the time of each injection, and the serum was tested for neutralizing antibody in tissue cultures. The details of injections and bleedings, together with antibody levels are shown in Table II.

Results. Propagation in small roller tubes. In cultures of monkey testis prepared with Mixture 199, but not infected with virus, fibroblasts grew out rapidly, and in about 2 to 3 weeks the lower third of the tube was lined by a confluent growth of cells. Cell multiplication continued throughout the 60-70 days that experiments were commonly run. In one series maintained for a longer period, cell division was observed after about 100 days. The pH of uninfected culture fluids was lowered regularly by the metabolizing tissue from 7.8 to about 7.0 in 4 days, for the first 12 to 15 fluid changes. Thereafter, the pH was lowered only to about 7.4. Microscopically, fibroblasts appeared normal for 60-70 days; slight degenerative changes were observed later, but these could not be mistaken for the more severe cytopathogenic changes induced by the virus. In infected cultures, all strains of the 3 types mentioned above induced, in some measure, cytopathogenic changes in the outgrowing fibroblasts. At one extreme, the Brunhilde strain produced extensive cytopathogenic changes, and cell multiplication ceased about 4 weeks after addition of virus. The Mahoney, MEF1, Y-SK, and Saukett strains likewise induced marked cytopathogenic effects. By contrast, in cultures infected with Lansing virus, cell multiplication and degeneration proceeded side by side. Thus, newly divided cells were found in close proximity to cells showing the minor degree of cytopathogenic effect usually associated with the growth of the Lansing virus in tissue culture. The Leon strain likewise induced only minor degrees of degeneration. The Canadian Eskimo strains had characteristics intermediate between those of the Brunhilde

TABLE I. Cultivation of Brunhilde Virus in Monkey Testis with Mixture No. 199, Second Subculture in Large Roller Tubes.*

Fluid changes	Age of cultures (days)	pH of pooled culture fluids on day of change†	Titer of pooled culture fluids (CPD)‡
1	9	7.45	2
2	13	7.45	4
3	17	7.5	3
4	21	7.6	4
5	25	7.6	5
6	29	7.6	5
7	33	7.65	4
8	37	7.65	5
9	41	7.65	3
10	45	7.65	4
11	49	7.75	2
12	52	7.75	3
13	56	7.75	2
14	59	7.8	1
15	63	7.8	1
16	66	7.8	0
17	73	7.8	0

* Inoculum was 50000 CPD of Brunhilde virus (4th fluid change of 1st subculture).

† Fluids were pooled from 2 roller tubes.

‡ Determined in tissue culture as described in text, and expressed as negative logarithms.

and Lansing strains. *Propagation in large roller tubes.* The Brunhilde strain was readily propagated in cultures of monkey testicular tissue treated with Mixture 199. The titers of the culture fluids, and the pH readings of the second subculture of virus are given in Table I. It was found that the pH did not drop as low in cultures in large tubes as in cultures in small tubes. Degenerative changes were observed in the outgrowing fibroblasts, and the appearances were essentially similar to those observed in cultures in small tubes. High titers of virus were produced in culture fluids for 52 days and smaller amounts up to 63 days. Lansing virus was also cultivated in large tubes. The LD_{50} values of the culture fluids, determined by the cerebral inoculation of mice (0.03 ml volumes), were between $10^{-1.0}$ and $10^{-2.0}$, *i.e.*, similar to those reported by others working with cultures in small tubes. *Antigenicity of tissue cultivated virus in monkeys.* Table II presents serum antibody titers in 2 groups of rhesus monkeys immunized with Brunhilde virus, one group having received monkey-passed virus and the other tissue-cultivated virus. It will be seen that 8/12 monkeys developed paralysis attributable to infection contracted from the first of the series of injections given every 2 weeks. None of the monkeys showed Brunhilde antibody before the start of immunization, but high titers were detected in samples removed at 4, 6, 8, 10, and 12 weeks, in both groups of monkeys. In the group immunized with tissue-cultivated virus, antibody titers of 3.0 or 4.0 had developed by the fourth week of the immunization program after only 2 injections of virus. The amounts of virus in these 2 injections were 2,000 and 200,000 CPD, respectively. In the group receiving monkey-passed virus approximately 40,000 PD_{50} of virus were given on each occasion, and these amounts did not appear to result in as brisk an antibody response. Although it is not possible to relate CPD to PD_{50} in absolute terms, it would appear likely that tissue cultivated virus is at least as potent antigenically as an equivalent amount of virus-infected cord tissue. The specific nature of the antibody response was indicated by the complete absence of neutralizing antibody to the Y-SK and Saukett strains in these sera. Satisfactory results were also obtained in monkeys immunized with Lansing tissue-cultivated virus on a similar schedule. Serum titers in individual monkeys were 3.0 or 4.0 at the fourth and sixth weeks. A recent short report by Salk(14) also refers to the employment of tissue cultivated viruses of the 3 antigenic types in the immunization of monkeys.

Conclusions. In experiments in which poliomyelitis virus was grown in cultures of monkey testis in roller tubes, it was shown that the commonly used feeding mixtures containing horse serum and embryo extracts could be replaced by the synthetic nutrient, Mixture No. 199, devised by Morgan, Morton, and Parker(5,6). Such cultures were found to support the growth of several different strains of poliomyelitis virus, and these produced cytopathogenic changes in the outgrowing fibroblasts. It was therefore possible to carry out titrations of viruses and of antisera in roller tube cultures of monkey testicular tissue with Mixture 199. In these titrations, an advantage of the use of Mixture 199 lay in the fact that no fluid changes needed

TABLE II. Serum Antibody Titers of Monkeys Immunized with Brunhilde Virus.

Type of inoculum	Monkey reference No.	No. of inoculations at 2-weekly intervals*	Serum antibody titers (CPID) at following stages of vaccination program† — Wk — 0	4	6	8	10	12	Outcome of vaccination program‡	Onset of paralysis wk
Monkey CNS	A612	5	0	—	2	2	3	—	NP	2
	613	1	—	—	—	—	—	—	FP	2
	614	1	—	—	—	—	—	—	FP	2
	615	5	0	—	2	2	3	3	NP	2
	616	6	0	2	3	4	4	4	H	
	617	2	—	—	—	—	—	—	FP	1
Tissue cultivated virus	A645	5	0	—	3	4	4	4	NP	1
	646	6	0	3	3	4	4	4	H	
	647	6	0	4	4	4	4	4	H	
	648	1	—	—	—	—	—	—	FP	1
	657	6	0	3	3	4	4	4	H	
	658	5	0	3	3	4	4	4	NP	1

* All injections given by intramusc. route, except first 2 inj. in A657 and A658 given subcut.; all inocula combined with equal parts of adjuvant; wt of cord/inj., .2 g; vol. of tissue culture fluid, 2 ml.

† Titration carried out in tissue culture; titers expressed as negative logarithms.

‡ NP = non-fatal paralysis; FP = fatal paralysis; H = healthy.

to be made during the 6 or 8 days that the titrations were run. The Brunhilde and Lansing strains were grown successfully in large roller tube cultures, and high titers were obtained. The Brunhilde and Lansing strains grown in such cultures stimulated satisfactory antibody levels in monkeys when incorporated with adjuvants and injected by the intramuscular route. The use of Mixture 199 is recommended for studies on the mechanism of the growth of poliomyelitis viruses in tissue culture. The usefulness of the synthetic medium as a nutrient in cultures infected with material derived directly from poliomyelitis patients is currently being investigated by us. Attempts are also being made to grow poliomyelitis viruses in bulk.

Summary. 1. The Brunhilde, Mahoney, Canadian Eskimo, Lansing, Y-SK, MEF1, Leon, and Saukett strains of poliomyelitis virus have been propagated in roller tube cultures of monkey testicular tissue treated with synthetic Mixture No. 199, devised by Morgan, Morton, and Parker. 2. Virus-induced cytopathogenic changes occurred in the fibroblasts and were similar to those observed in cultures treated with mixtures containing naturally occurring ingredients. 3. Virus and serum titrations in roller tube cultures were read 7 days after infection of the cultures; during this period no change of culture fluid was necessary. 4. The Brunhilde and Lansing strains were propagated in large roller tube cultures treated with Mixture 199, and high titers were obtained. 5. Culture fluids infected with Brunhilde and Lansing viruses from large roller tubes (mixed with adjuvants) were used to immunize groups of monkeys by the intramuscular route, and high serum antibody levels (4-0) developed promptly.

We are indebted to Dr. R. C. Parker and his colleagues for supplies of the synthetic medium. We wish to thank Doctors D. Bodian, H. A. Howe, J. L. Melnick, and J. E. Salk for sending us material representing various strains of poliomyelitis virus.

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