IN VITRO DIFFERENTIATION OF VIRULENT AND ATTENUATED POLIOVIRUSES BY THEIR GROWTH CHARACTERISTICS ON MS CELLS*, ‡

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PLATES 1 TO 3

(Received for publication, August 19, 1958)

This paper is concerned with the correlation of an *in vitro* marker of poliovirus particles, the capacity for multiplication on a monkey stable (MS) cell line, with the degree of neurovirulence for monkeys. Vogt *et al.* (1) discovered that the plaque formation of most attenuated strains of poliovirus is delayed or inhibited, when the amount of bicarbonate in the agar overlay is reduced. However, a number of attenuated strains of the delayed, or *d*, type have been shown to be capable of reversion to the wild, d^+ type, without markedly changing their low degree of neurovirulence for monkeys (2, 3).

The present authors have recently discovered another marker which may be used to differentiate poliovirus particles. Virulent strains grow well on primary monkey kidney cultures and on the MS cell line.[1] Attenuated strains grow poorly and form very tiny plaques, or none at all, on MS cells. They are classified as *MS* mutants, and are said to possess the *MS* character, in contrast to the MS^+ marker of wild, virulent polioviruses.

Materials and Methods

Virulent Poliovirus Strains.—Type 1 (Mahoney strain), Type 2 (MEF_1 strain), and Type 3 (Saukett strain) used in the preparation of poliomyelitis vaccine. All had been purified by plaque passage and had been used for work on their *d* character (3). In addition we included a Type 1 strain (Baylor-1) isolated in the spring of 1958 from the spinal cord of a fatal case of poliomyelitis in a triply vaccinated person.

* Aided in part by a grant from The National Foundation

‡ We are happy to have this paper included as one of a number dedicated this month to Professor E. Berger, Kinderspital, Basel, Switzerland, on the occasion of his 60th birthday.

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[1] This line was kindly made available to us by Dr. Alfred Tytell, Merck, Sharp, and Dohme, West Point, Pennsylvania.

Attenuated Poliovirus Strains.—Three strains, LSc (Type 1), Y-SK (Type 2), and Leon (Type 3) had also been plaque-purified and used in previous studies (3). In addition, three strains plaque-purified and described by Sabin (4) were included. Dr. Sabin kindly sent us frozen aliquots of the large 25-liter pools, prepared at Merck, Sharp, and Dohme, December, 1956 (4). They were used without further passage.

Newly Isolated Strains of Type 3 Poliovirus.—Sixteen strains in their first few monkey kidney passages were kindly made available by Dr. Karl Habel. These strains were selected for study here because 8 had been isolated from patients with paralytic poliomyelitis and 8 from patients with aseptic meningitis.

Cell Cultures.—Primary monkey and human kidney cultures were prepared using 0.5 per cent lactalbumin enzymatic hydrolysate—2 per cent calf serum medium (5). HeLa cultures (6) were grown in medium 199–10 per cent calf serum. MS cells benefitted by the addition of tryptose phosphate broth (7). Stock cultures were grown for 6 to 7 days in MS-1 medium consisting of 70 per cent 199-Earle's solution, 20 per cent tryptose phosphate broth, and 10 per cent calf serum. (MS cells also grow well if the 199 component of the medium is replaced with a 0.5 per cent lactalbumin hydrolysate medium in Earle's salt solution, fortified with glutamine, 0.375 gm. per liter, and glucose, total 2 gm. per liter.) After the cultures formed a monolayer, they were treated with 0.2 per cent trypsin, sedimented at 350 R.P.M. for 3 minutes, and resuspended in MS-1 medium. For seeding plaque bottles, 7 ml. of suspension containing approximately 100,000 cells per ml. were placed in a 2 ounce flat prescription bottle. At the end of 3 to 4 days at 37°C., the growth medium was removed and replaced with fresh MS-1 medium. When the outgrowth of the monolayer sheet was confluent, usually in an additional day, the cultures were ready for use. Tube cultures were prepared with 0.5 ml. of the suspension. They were used after 1 or 2 days of cultivation.

Cell Counting.—For total cell counts, 0.1 per cent crystal violet in 0.1 M aqueous citric acid was used. For counting live cells, 0.05 ml. of a 0.01 per cent solution of neutral red was added to 1 ml. of cell suspension and the mixture incubated at 37°C. for 20 minutes. Dead cells were counted by mixing 0.05 ml. of a 1.0 per cent trypan blue solution with 1 ml. of cell suspension. The cell count was done immediately, because trypan blue is toxic to cells.

Virus Titrations.—Plaque-forming units (PFU) and TCD_{50} titers were assayed as described (8, 9). Calf serum used in all maintenance media had been pretested to be certain that it was free of inhibitors of polioviruses.

The procedures with the MS cells had to be modified slightly. For tube titrations in MS cultures, the concentration of calf serum was decreased to 1 per cent, and 0.25 per cent bovine albumin added to the 199-tryptose phosphate broth medium. The plaque assay method on MS cells had to be modified to provide a thin layer of agar with a relatively high concentration of neutral red. When the cells in the bottles had grown into a complete monolayer, the nutrient fluid was removed, and virus samples of 0.2 ml. were introduced. After an adsorption period of 50 minutes, 4 ml. of melted MS agar overlay (formula below) was layered over the culture. After 30 minutes during which the agar overlay solidified, the bottles were turned over and then transferred to the 37° incubator.

MS Agar Overlay.—*A. Nutrient medium:* Earle's solution (10× concentrated without either phenol red or $NaHCO_3$), 18.0 ml; sterile distilled water, 61 ml.; bovine albumin (5.5 per cent stock solution), 3.6 ml.; $NaHCO_3$ (7.5 per cent stock solution), 5.4 ml.; neutral red, (1 per cent), 0.35 ml.; antibiotics (1 ml. of penicillin, containing 40,000 units/ml.; 0.2 ml. of streptomycin, containing 200 mg./ml.; and 0.4 ml. of mycostatin, containing 50,000 units/ml).

B. Agar solution: Agar, 2.7 gm.; distilled water 90.0 ml. After sterilization, it was cooled to 45°C. before use.

C. Mixture: Just before making the overlay, equal portions of *A* (at 37°C.) and *B* (at 45°C.) were mixed.

Harvesting of Virus.—The supernatant fluid (extracellular virus) was removed from the infected bottles and centrifuged for 15 minutes at 1000 R.P.M., in order to remove any cells that had come loose from the glass. It was then frozen at −20°C. and stored until titrated by the plaque technique. After the supernatant fluid had been removed, the cells were scraped from the glass with a rubber-tipped glass rod. They were then resuspended in 7 ml. of chilled medium and frozen for titration of intracellular virus. Before assay, cells were disrupted by 3 cycles of freezing and thawing.

Test for d Character.—This character was determined by measuring the reduced efficiency of plating (E.O.P.) under a low bicarbonate agar overlay on monkey kidney cells. In all tests, the virulent Mahoney strain and the attenuated LSc strain were included as controls, and PFU per milliliter were compared in bottle cultures with 0.1 per cent and 0.4 per cent $NaHCO_3$ in the agar overlay (3).

Test for MS Character.—This character was determined by measuring the plaque size on MS cells, after 6 days of incubation. Virus strains that produced tiny plaques smaller than 1 mm. in diameter, or no plaques at all, were classified as *MS*. On the other hand, strains that produced larger plaques on MS cells were considered to possess the MS^+ character of wild strains. Again, the virulent Mahoney strain (at a tissue culture fluid concentration of 10^{-5}) and the attenuated LSc strain (at a tissue culture fluid concentration of 10^{-3}) were included as controls in each test.

EXPERIMENTAL

Growth of Polioviruses in Tube Cultures.—Virus stocks for these experiments were grown on primary monkey kidney cells. For the tube titrations, the cytopathogenic endpoints were calculated from the 6th day reading. Results are shown in Table I and Text-figs. 1, 2, and 3. There was no significant difference of titers between virulent and attenuated viruses in cultures of primary monkey kidney cells, HeLa cells, or human kidney cells, although there was a delay in the appearance of cytopathogenicity and a slight decrease in sensitivity with the human kidney cells. The MS cells showed a decreased susceptibility, which was very marked for the attenuated polioviruses of all three types. There was a 100-fold difference in titer between the virulent and attenuated viruses when they were tested in MS cells. An exception was noted: the Saukett strain, considered to be a virulent Type 3 strain, also showed restricted growth on the MS cells.

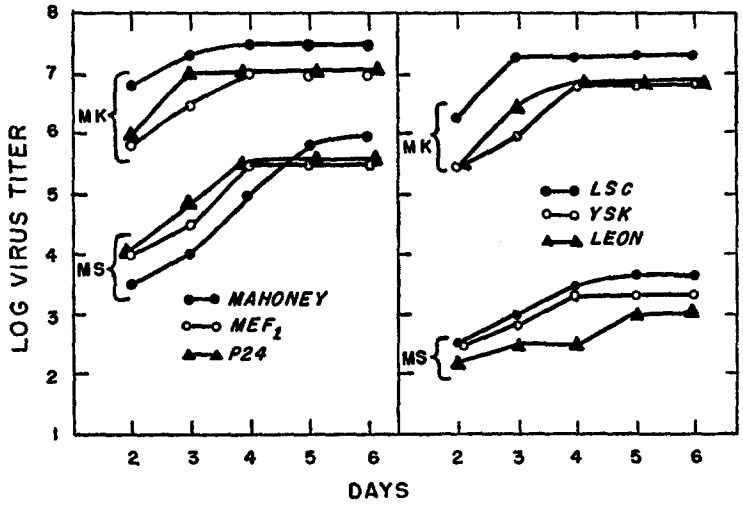
The cytopathogenic changes of MS cultures were similar to those of freshly trypsinized monkey kidney (MK) cells, except that the degenerated cells were not as round nor as refractile as in MK cultures. Also, the degenerated MS cells had a greater tendency to clump together (see Figs. 1 and 2).

Growth of Polioviruses in Plaques under Agar.—When studied by the plaque method on MS cells, polioviruses could be separated into two groups. As shown in Table II, virulent strains produced plaques 3 to 5 mm. in diameter, while the attenuated strains produced plaques smaller than 1 mm. in diameter or no visible plaques at all (see Fig. 3). The attenuated strains also yielded

TABLE I

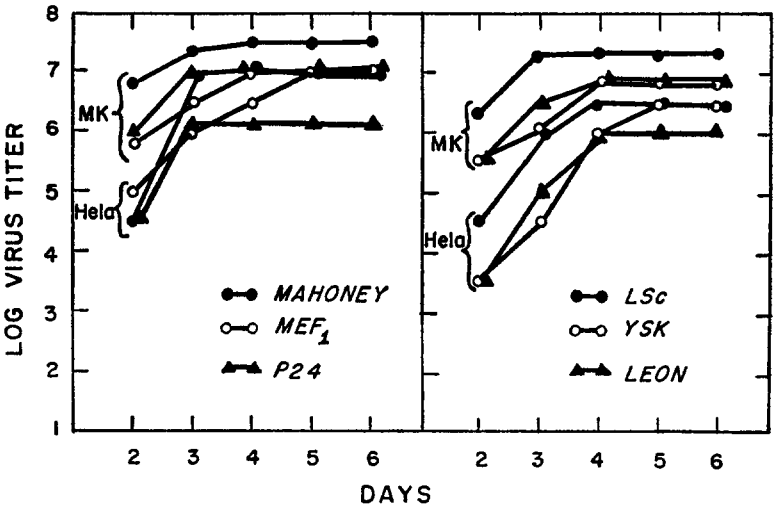
Titrations of Polioviruses in Tube Cultures of Monkey Kidney (MK), Monkey Stable (MS), HeLa, and Human Kidney (HK) Cells

Virus (grown on MK cells)	Neuropathogenicity	On MK cells (neg. log TCD_{50})	Log diff. vir.-att.	On MS cells (neg. log TCD_{50})	Log Diff. vir.-att.	On He-La cells (neg. log. TCD_{50})	Log diff. vir.-att.	On HK Cells (neg. log TCD_{50})	Log. diff. vir.-att.
Type 1, Mahoney...	Virulent	7.5		6.2		7.0		7.5	
LSc.....	Attenuated	7.3	0.2	3.7	2.5	6.5	0.5		
Sabin's LSc.....	Attenuated	7.0	0.5	4.0	2.2			7.0	0.5
Type 2, MEF_1.....	Virulent	7.0		5.8		7.0		7.3	
YSK.....	Attenuated	6.7	0.3	3.3	2.5	6.5	0.5		
Sabin's P712.....	Attenuated	6.5	0.5	3.5	2.3			5.8	1.5
Type 3, P24.....	Virulent	7.0		5.5		6.2		6.8	
Saukett.....	Virulent (?)	7.0	0.0	3.0	2.5	6.2	0.0		
Leon.....	Attenuated	6.8	0.2	3.0	2.5	6.0	0.2		
Sabin's Leon.....	Attenuated	6.8	0.2	3.3	2.2			6.0	0.8

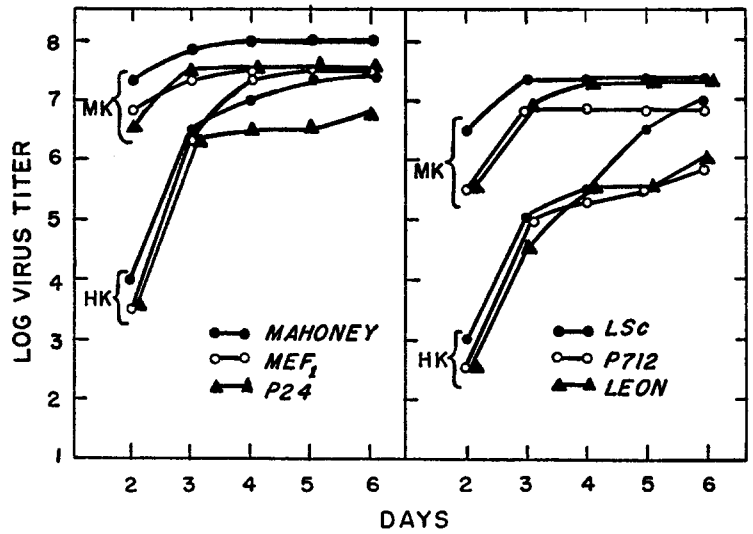


TEXT-FIG. 1. Comparative titrations of virulent (left) and attenuated (right) strains of poliovirus in tube cultures of primary monkey kidney (MK) and of monkey stable (MS) cells.

much lower titers. The results of three different experiments are shown. In two, virulent Mahoney strain had a PFU titer more than 100 times higher than attenuated LSc, when grown on MS cells, and in the third test, the titer was over a million times greater. In contrast, there was only a slight difference



TEXT-FIG. 2. Comparative titrations of virulent (left) and attenuated (right) strains of poliovirus in tube cultures of primary monkey kidney (MK) and of HeLa cells.



TEXT-FIG. 3. Comparative titrations of virulent (left) and attenuated (right) strains of poliovirus in tube cultures of monkey kidney (MK) and of human kidney (HK) cells.

(less than fourfold) in PFU titer on MK cells (see Fig. 4). Again, the Saukett strain was observed to behave like the attenuated ones.

In the two right-hand columns of Table II, results of control plating on MK cells at high bicarbonate concentration are shown. In these control results,

no significant difference could be demonstrated, either in PFU titers or in plaque size, which could distinguish between virulent and attenuated polioviruses.

Kinetics of Multiplication of Poliovirus in MS Cell Monolayers.—In an attempt to elucidate the processes responsible for the restricted growth of attenuated polioviruses on MS cells, the following experiments were designed. First a comparison was made of the adsorption of polioviruses to MK and to

TABLE II

Plaque Formation of Polioviruses on MS and MK Cells

Virus (grown on MK cells)	Neuropathogenicity	On MS cells						On MK cells	
		(Log PFU/ml.)*			Plaque size (average diameter in mm.)			(Log PFU/ml.)	Plaque size (average diameter in mm.) 6th day reading
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3		
Type 1, Mahoney	Virulent	7.7	7.2	7.3	3–4	3–5	3–5	8.4	14–18
Baylor-1	Virulent	7.0	7.2		3–5	3–5		7.8	13–16
LSc	Attenuated	5.6	5.0	0	0–1	0–1	0	8.0	14–17
Sabin's LSc	Attenuated	0	0	0	0	0	0	7.8	12–15
Type 2, MEF_1	Virulent	5.8	7.0		3–5	3–5		8.0	14–16
YSK	Attenuated	4.4	4.8		0–1	0–1		7.3	14–16
Sabin's P712	Attenuated	0	0		0	0		7.3	9–11
Type 3, P24	Virulent	7.0	7.2		4–6	4–6		8.0	14–16
Saukett	Virulent (?)	5.0	5.2		0–1	0–1		7.7	13–15
Leon	Attenuated	3.5	0		0	0		7.3	13–15
Sabin's Leon	Attenuated	0	0		0	0		7.5	10–12

* If 10^6 or more particles of attenuated (MS) virus are seeded on a monolayer, the cell sheet often undergoes complete degeneration without the appearance of plaques. Titers of attenuated strains on MS cells are difficult to establish, unless the plaques grow to a size of at least 1 mm.

MS cells, using methods already described (10). As shown in Table III, the virulent and attenuated polioviruses adsorbed equally well to the MS cells. There was no difference in efficiency of adsorption of virus by MK or MS cells.

The growth curves of virulent and attenuated Type 1 poliovirus in MS cell monolayers were investigated. Virus stocks grown in MK cells and used for the above experiments, were seeded into 2-ounce bottle cultures of MS cells. The procedures were essentially like those previously used in this laboratory. After an adsorption period of 1 hour at 37°C., the cultures were washed three times to remove residual virus, and maintenance medium was added. At intervals, as shown in Text-figs. 4 and 5, a bottle from each series was removed, and the virus in the medium was titrated for PFU content on MK cells.

The cells were rinsed to remove extracellular virus, then scraped and collected in the same volume of medium as had been present in the culture. The preparation was frozen and thawed three times, and then titrated for intracellular virus. The growth curves in Text-fig. 4, for Mahoney virus, indicated a response like that previously obtained in primary MK cultures (10, 11). Intracellular virus was rapidly produced, and this was followed by its release from cells and the subsequent appearance of cytopathic changes (12). In contrast, the results with the attenuated LSc strain showed that the amount of virus formed, intracellular and extracellular, was considerably less than that produced by the virulent Mahoney strain, even when the same multiplicity of infection was

TABLE III

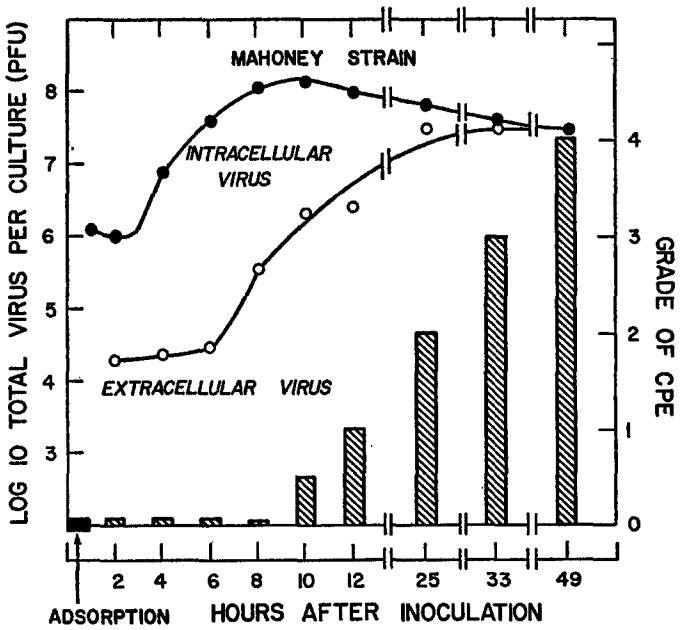
Comparison of the Adsorption of Poliovirus on MK and MS Cells after a Contact Period of 1 Hour at 37°C.

Type of cell	Virus	Input multiplicity (PFU/cell)	Multiplicity after removing free virus (PFU/cell)	Virus adsorbed
				per cent
MK	Mahoney	1.0	0.5	50
		0.1	0.02	20
MS	Mahoney	2.0	0.8	40
		0.2	0.06	30
	LSc	3.2	0.6	20
		0.2	0.1	50
			0.08	40

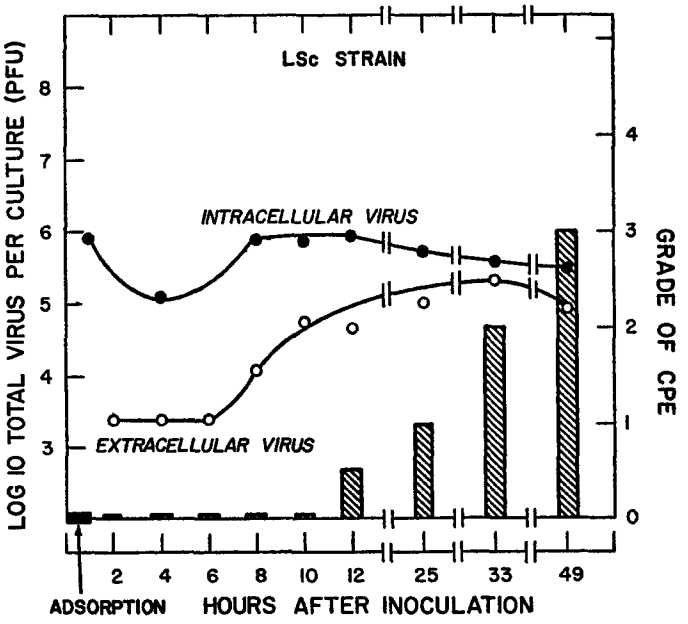
used. Even though the virus yields differed, the average maturation time (the time required to produce 50 per cent of the total virus) of both strains was about 7 hours after inoculation. Virulent Mahoney strain produced faster and more complete cytopathic changes than did the attenuated LSc strain.

The yield of virus was calculated for the MS cell, by determining the average number of cells in the bottle cultures used in the experiments and the maximum amount of infectious virus yielded during the growth cycle. It can be seen from the data in Table IV that the MS cells yielded about the same amount of virulent virus as do MK cells, when virus yields were estimated by the same procedure (13). However, the yield of attenuated virus was only 0.5 per cent of this value.

Growth of Freshly Isolated Strains of Type 3 Poliovirus.—Following the results with the supposedly virulent Saukett virus, which possessed the *MS* character, a more extensive investigation was made of Type 3 strains. It is well known that the Saukett strain possesses a lesser degree of neurovirulence than the Mahoney and MEF_1 strains. Furthermore, the Saukett strain was a



TEXT-FIG. 4. Intracellular and extracellular growth curves of virulent Mahoney virus in MS cells. Plaque assays were carried out on MK cells, as they were for the data in Text-fig. 5.



TEXT-FIG. 5. Intracellular and extracellular growth curves of attenuated LSc virus in MS cells.

plaque-purified derivative, and an attenuated variant might have been selected by this procedure. Consequently a study of 16 wild Type 3 viruses was made. These strains, isolated in Dr. Karl Habel's laboratory, had been passed

TABLE IV

Yields of Virulent and Attenuated Polioviruses per MS Cell

Strain	Multiplicity of infection (PFU/cell)	No. of cells per culture	Maximum yield,* (PFU/culture)	Calculated yield (PFU/cell)
Virulent Mahoney	0.6	$10^{6.2}$	$10^{8.2}$	100
	0.06	$10^{6.0}$	$10^{8.1}$	130
Attenuated LSc	0.6	$10^{6.0}$	$10^{5.7}$	0.5
	0.08	$10^{6.2}$	$10^{5.9}$	0.5

* Obtained from plotting growth curves, see Text-figs. 4 and 5.

TABLE V

Growth of Wild Strains of Type 3 Poliovirus Freshly Isolated from Paralytic and Non-Paralytic Patients

Clinical form	Strain	Log titer, PFU/ml.		Plaque size on MS cells (*diameter in mm.*)	*MS* character	*d* character
		On MK cells	On MS cells			
Paralytic	P24	6.4	5.2	3–5	MS^+	d^+
	P28	6.7	4.5	<1	*MS*	d^+
	P33	6.5	5.7	3–5	MS^+	d^+
	P45	6.0	4.7	1–2	MS^+	d^+
	P131	6.7	5.5	2–4	MS^+	d^+
	P137	6.0	4.0	<1	*MS*	d^+
	P145	6.7	3.5	<1	*MS*	d^+
	P161	6.2	3.5	<1	*MS*	d^+
Non-paralytic	N2687	8.2	5.0	<1	*MS*	d^+
	N2858	8.0	5.0	<1	*MS*	d^+
	N2889	7.8	5.0	<1	*MS*	d^+
	N2899	7.7	5.7	<1	*MS*	d^+
	N3129	7.7	4.4	<1	*MS*	d^+
	N3280	7.5	5.6	<1	*MS*	d^+
	N3850	7.5	4.0	<1	*MS*	d^+
	N3986	7.5	5.2	<1	*MS*	d^+

once or twice in MK cultures before they were typed. We tested them for *MS* and *d* characters; the results are shown in Table V.

Strains which produced large plaques were obtained only from the group of paralytic patients, 4 of 8 such strains being MS^+. However, all 16 strains

possessed the d^+ character, for all grew equally well under agar at low and high concentrations of bicarbonate.

In contrast to certain of the highly attenuated strains which had failed to produce visible plaques on MS cells (see Table II), the 12 *MS* strains studied here all yielded plaques even though of the minute variety.

As a result of this study, the P24 strain which produced large plaques, and the N2858 strain which produced minute plaques were selected as prototypes of MS^+ and *MS* strains, respectively (see Fig. 5). They were then tested for neurovirulence, as described below.

Relationship of Monkey Neurovirulence to MS and d Characters.—The results of tests on the new Type 3 strains, for neurovirulence in monkeys, are shown

TABLE VI

Correlation of Neurovirulence and MS and d Characters of Newly Isolated Type 3 Strains

Virus	*In vitro* markers	Intraspinal virulence (10^5 PFU in 0.1 ml. inoculum)		Intracerebral virulence (10^6 PFU in 1 ml. inoculum)		Intramuscular virulence (10^8 PFU in 2 ml. inoculum)	
		Paralysis	CNS lesions	Paralysis	CNS lesions	Paralysis	CNS lesions
P24	MS^+d^+	2/2*	2/2*	2/2	2/2	4/4	4/4
N2858	$MS\ d^+$	2/2	2/2	0/2	2/2 (mild)	0/4	0/4

* 2/2: numerator, monkeys developing paralysis or CNS lesions, as indicated; denominator, monkeys inoculated.

in Table VI. Monkeys were inoculated intraspinally with 10^5 PFU as the most severe test; all proved pathogenic and no difference could be seen with a test of this degree of sensitivity. With the intracerebral test, again using a challenge dose of 10^5 PFU, only the MS^+ virus produced paralysis and extensive lesions within the brain and spinal cord. The *MS* virus failed to produce any clinical signs in the monkey, although mild focal lesions were found in the CNS. The inoculation of as many as 10^8 PFU of the *MS* virus in the muscles failed to produce paralysis or lesions, but the same dose of MS^+ virus caused paralysis and extensive lesions in all 4 monkeys inoculated.

Another series of tests[2] was carried out with Sabin's attenuated strains (Dec., 1956 lot), and the results are listed in Table VII. The three strains are at the low end of the spectrum so far as the *MS* character is concerned, in that none of them produced visible plaques. The findings on neurovirulence, with paralysis occurring only with large doses of virus injected intraspinally, are confirmatory of Sabin's results (4).

[2] The full details of these and related experiments on the properties of attenuated polioviruses after passage through monkeys will form the basis of a report to be made together with Dr. Gerald L. Van Hoosier, Jr.

It must be emphasized that these attenuated strains are not devoid of neurotropism. Even though only 3 of 8 monkeys inoculated intraspinally

TABLE VII

Comparison of Neurovirulence and in Vitro Characters of Sabin's Attenuated Strains (Dec., 1956 Lot)*

Virus‡	*In vitro* markers	Intraspinal virulence ($10^{6.0}$ PFU)		Intracerebral virulence ($10^{7.0}$ PFU)		Intramuscular virulence ($2 \times 10^{7.0}$ PFU)	
		Paralysis	CNS lesions	Paralysis	CNS lesions	Paralysis	CNS lesions
LSc	*MS d*	3/8	7/8	0/8	1/8 (mild)	0/8	2/8 (mild)
P712	*MS d*					0/8	0/8
Leon	*MS d*					0/8	0/8

* Cortisone was injected into half of the monkeys in each group. The dose was 100 mg. intramuscularly on days 0, 2, 4, 6, and 8. Because there was no difference in the results of the group receiving cortisone and that receiving none, the two groups have been consolidated in the results shown in this table.

‡ The three attenuated strains yielded titers between $10^{7.0}$ and $10^{7.5}$ PFU per ml.

TABLE VIII

Summary: Comparison of Neurovirulence, d Character, and MS Character

Virus	*In vitro* character	Intracerebral virulence*		Intramuscular Virulence: Per Cent Showing Paralysis or CNS Lesions
		Paralysis	CNS Lesions	
Type 1, Mahoney	MS^+d^+	2/2	2/2	55–100‡
LSc	*MS d*	0/2	0/2	0
Sabin's LSc	*MS d*	0/8	1/8 (mild)	25 (mild lesions only)
Type 2, MEF_1	MS^+d^+	2/2	2/2	50–95‡
Y-SK	*MS d*	0/2	0/2	0
Sabin's P712	*MS d*			0
Type 3, P24	MS^+d^+	2/2	2/2	100
Saukett	$MS\ d^+$	0/2	2/2 (mild)	10–20‡
Leon	*MS d*	0/2	0/2	0
N2858	$MS\ d^+$	0/2	2/2 (mild)	0
Sabin's Leon	*MS d*			0

* Challenge dose of 10^5 PFU or greater.

‡ These data were obtained from a large collaborative study carried out by the manufacturers of poliovaccine. The inocula were the strains as indicated, but each manufacturer used his own seed virus, rather than the plaque-purified derivative used for our studies.

became paralyzed, 7 of the 8 developed lesions in the CNS. In 5 of the 7 CNS-positive monkeys, the lesions were restricted to the lumbar portion of the cord, close to the injected area.

Additional correlative data, together with a summary of data in Tables VI and VII, are presented in Table VIII. The three MS^+d^+ strains, Mahoney, MEF_1, and P24, are characterized by their neuropathogenicity when inoculated by the intracerebral or intramuscular routes. The attenuated, *MS d* strains have had the least neurotropism, with the *MS* d^+ strains giving either low or intermediate responses of neurovirulence. Although the *MS* d^+ strains were not paralytogenic at an intracerebral challenge dose of 10^5, they did produce lesions in the CNS, although of a characteristically mild type. The results of the intramuscular inoculations carried out in the course of this study corroborate the tests by the other routes. It is worth noting that the

TABLE IX

Effect of Passage of Virus in MS Cells

Strain	Character of MK-grown virus	No. of passages in MS cells	Character of virus in last MS passage	Intracerebral neurovirulence
Mahoney	MS^+d^+	12	MS^+d^+	2/2*
LSc	*MS d*	5	*MS d*	
MEF_1	MS^+d^+	12	MS^+d^+	2/2
Y-SK	*MS d*	5	*MS d*	
Saukett	*MS* d^+	12	*MS* d^+	
Leon	*MS d*	5	*MS d*	

* 2/2 = 2 paralyzed, of 2 inoculated with 10^5 PFU.

Mahoney, MEF_1, and Saukett seed pools used by the manufacturers of Salk vaccine exhibit neuropathogenic properties in keeping with their *in vitro* markers. The two MS^+d^+ strains produced at least 50 per cent paralysis, but the Saukett strain (*MS* d^+) yielded only 10 to 20 per cent positives when undiluted tissue culture fluid was used as the challenge dose.

Effect of Passage in MS Cells on Neurovirulence of Virulent and Attenuated Viruses.—Six strains were passed for 5 or 12 serial passages in MS cultures. After this, they were retested for the *MS* and *d* characters, and two of the virulent strains were also retested in monkeys. The results, shown in Table IX, indicate that, as far as these passages went, the viruses proved to be stable genetically.

DISCUSSION

The present work demonstrates that polioviruses may be classified by the extent to which they grow in MS cells, and that this property seems to be correlated with the degree of neurovirulence of the virus particles. As with the *d* marker of Vogt *et al.* (1), the *MS* character is associated with attenuated strains,

in contrast to the d^+ and MS^+ markers of wild, virulent strains. The fact that *d* viruses may revert to d^+ without showing an increase in neurovirulence indicates that other genetic characters are involved, and might well explain the degrees of virulence exhibited by different lines of even the same strain of virus (1–4).

The growth patterns of virulent and attenuated strains indicated that in MS cells the attenuated strains yielded titers as low as 0.01 per cent of the values obtained in MK cultures. There were hardly any differences with titers of virulent strains in the different cell types.

Plaque size is another marker of poliovirus, and some attenuated strains have been reported to produce small plaques on MK cells (14–16). However, small plaque size on MK monolayers is not a general property associated with attenuation, as clearly evidenced in Fig. 4.

The finding that the Saukett strain possesses the *MS* character is in keeping with its low degree of neurovirulence for the monkey. Our data on the mildness of Saukett virus confirm the results already published by Bodian (17). It is noteworthy that this strain is d^+, and possesses a higher degree of neuropathogenicity than *MS d* strains like LSc. In the course of this study, 16 new strains belonging to Type 3 were screened for their *MS* and *d* markers. Two were selected for neurovirulence tests: the P24 strain, MS^+d^+, proved to be highly virulent, similar to the Mahoney strain in the high degree of paralysis produced even after intramuscular inoculation; the N2858 strain, *MS* d^+, behaved like the mild Saukett virus which possessed the same set of markers.

As in a previous study (3), a strain was considered attenuated if it failed to be paralytogenic for the monkey if at least 10^5 PFU were inoculated intracerebrally. Strains characterized *in vitro* as *MS* behaved in this way. It must be emphasized that this is an arbitrary decision, and that some strains giving the test of an MS virus (*i.e.*, plaque size of <1 mm.) may be paralytogenic if the challenge dose contains more than 10^5 PFU.

We consider the problem of *in vitro* markers of the polioviruses associated with virulence or attenuation to be of more than academic interest because at the present writing thousands of children are being fed attenuated polioviruses, as a possible live virus vaccine. With such trials increasing in number in normal population groups, it seems essential to learn more about the genetic stability of these viruses in the course of their multiplication in the human enteric tract, and it is obviously impossible to carry out the desired surveillance in monkeys, because sufficient facilities do not exist in any laboratory.

SUMMARY

The *MS* character of poliovirus particles is described. Strains that produced relatively large plaques, up to 6 mm. in diameter, on a monkey stable (MS)

cell line, were classified as MS^+. Such strains were found to include the highly virulent poliovirus strains. Attenuated strains appeared deficient in this gene, for they produced tiny plaques less than 1 mm. in size, or no visible plaques at all; they were considered as *MS* mutants.

Strains of maximum neurovirulence for the monkey possessed the MS^+ and d^+ characters, while those of greatest attenuation were *MS d*. Strains possessing the *MS* d^+ character showed high or intermediate attenuation.

Virulent strains grew equally well in MS or primary monkey kidney (MK) cells. Attenuated strains gave lower titers in MS cultures than in MK cultures. While MS cells after infection with a virulent virus yielded about 100 to 200 PFU per cell, only one PFU per MS cell was detected after infection with an attenuated virus.

A study of newly isolated Type 3 strains showed them to consist of the MS^+d^+ or *MS* d^+ type. The MS^+d^+ virus proved to be highly paralytogenic for monkeys even by the intramuscular route, while the *MS* d^+ virus was of the partially attenuated type.

The study of *in vitro* characters of viruses from children fed attenuated poliovirus offers a possibility for following genetic changes of the viruses after multiplication in the human enteric tract.

The authors wish to acknowledge with thanks the kind assistance of Dr. Gerald L. Van Hoosier, Jr., and Dr. Ruth Kirchstein, for the monkey testing and histopathological examinations associated with the neurovirulence studies.

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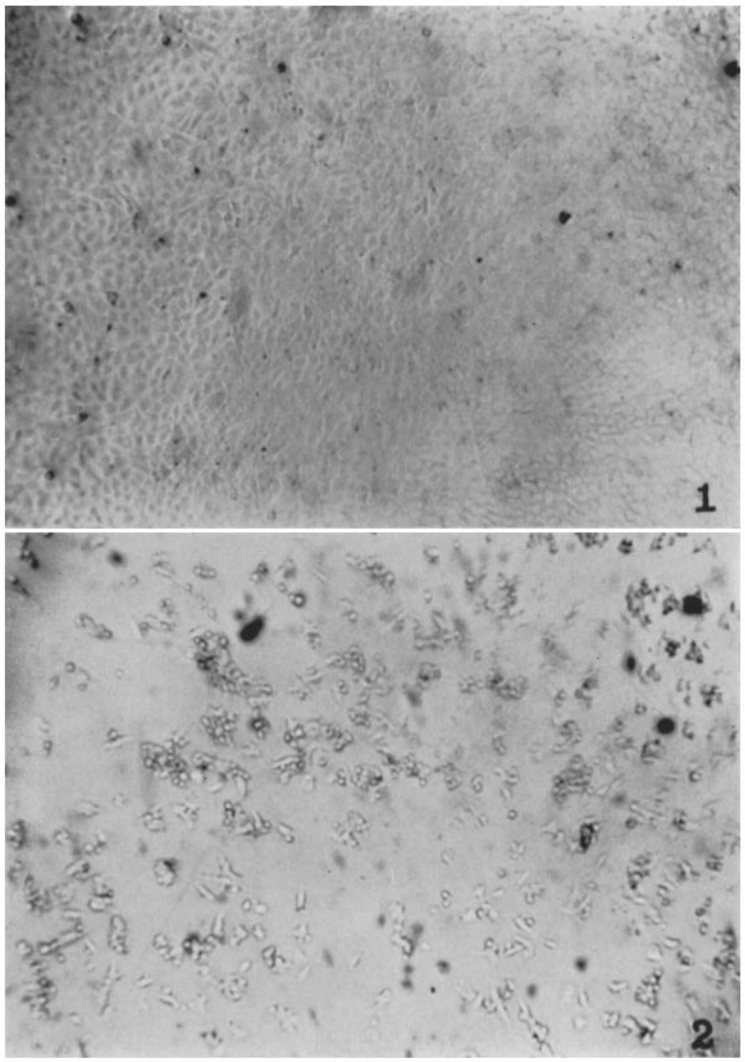
EXPLANATION OF PLATES

PLATE 1

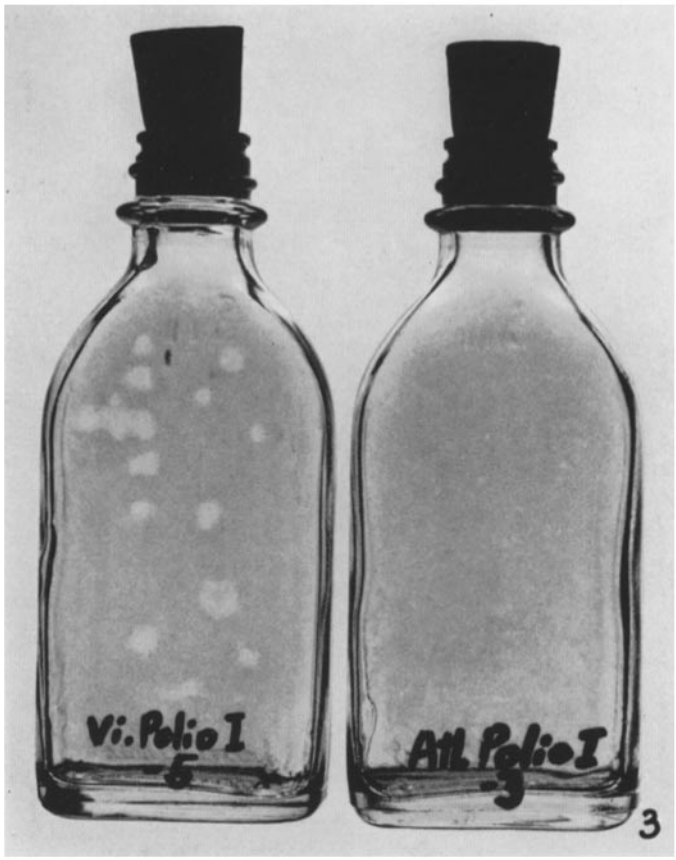
FIG. 1. Normal MS cell culture, unstained, × 35.
FIG. 2. MS cell culture infected with virulent Mahoney poliovirus, unstained, ×35.



(Kanda and Melnick: Virulent and attenuated polioviruses)

PLATE 2

FIG. 3. Plaques of Type 1, virulent Mahoney strain, and attenuated LSc strain, on MS cells 6 days after seeding. Concentration of tissue culture fluid: Mahoney, 10^{-5}; LSc, 10^{-3}.

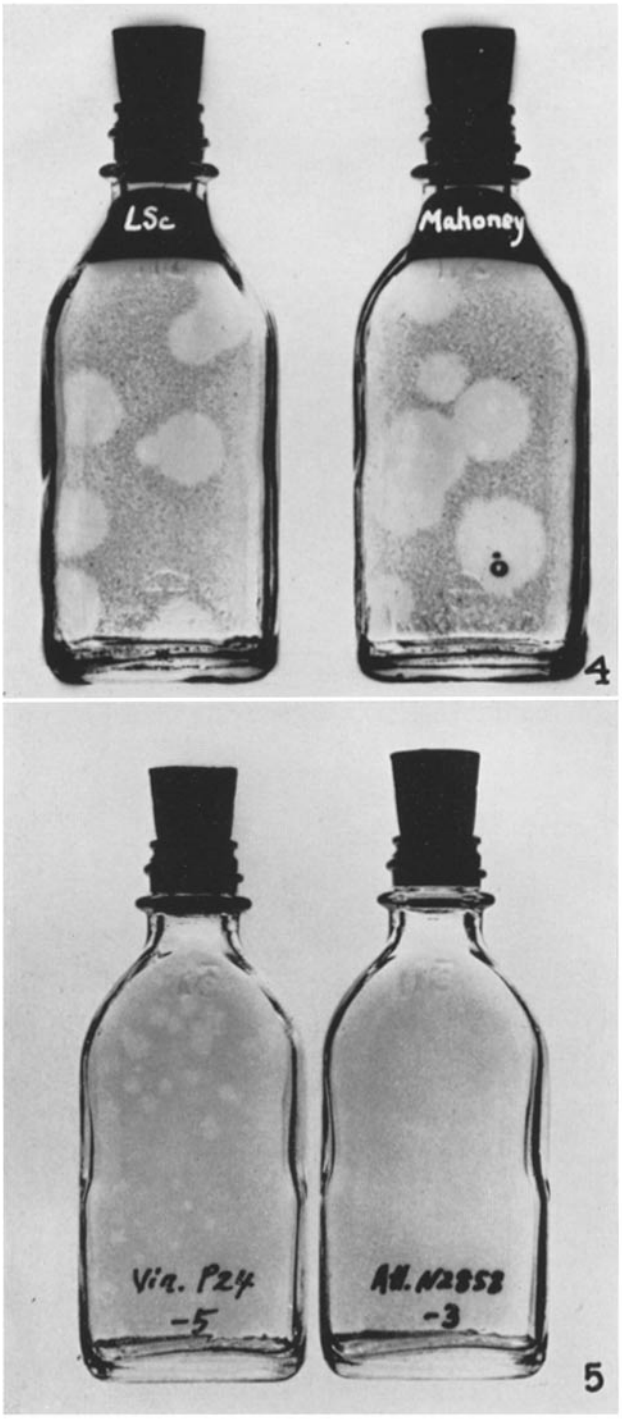


(Kanda and Melnick: Virulent and attenuated polioviruses)

PLATE 3

FIG. 4. Plaques of virulent Mahoney strain and attenuated LSc strain on MK cells. Monolayers had been seeded 4 days earlier with infectious tissue culture fluid at a concentration of 10^{-7}.

FIG. 5. Plaques of Type 3, virulent P24 strain and attenuated N2858 strain, on MS cells 6 days after seeding. Concentration of tissue culture fluid: P24, 10^{-5}; N2858, 10^{-3}.



(Kanda and Melnick: Virulent and attenuated polioviruses)