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Variation in Virulence of Poliovirus. III. On Type III Virus by Plaque Method.* (24034)

C. P. LI AND W. G. JAHNES

Division of Biologics Standards, National Institutes of Health, Bethesda, Md.

In a previous paper(1) experimental variation in mouse virulence of type III (Leon) poliovirus grown in tissue culture (TC) was reported and the influence of host cell and maintenance medium on viral virulence was demonstrated. The experiments have been extended, using the plaque technic. A mixed virus population and one purified by terminal dilution and by the plaque method were studied in regard to (a) distribution of virulent and avirulent progeny, and (b) reversibility of avirulent phase under influence of virulence enhancing media. The question as to whether the avirulent variant failed to multiply in the host or whether it multiplied without causing paralysis was also studied. The results are here reported.

Materials and methods have been described, (1) except for the following additions or modifications. The *plaque technic* of Dulbecco and Vogt(2,3) as modified by Hsiung and Melnick(4,5) was followed. Rhesus monkey kidney tissue cultures (MKTC) grown for approximately one week in Melnick's medium (0.5% lactalbumin hydrolysate in Hanks' salt solution with 2% calf serum), in 2-oz flat "prescription" bottles, were used. After removing the medium, each bottle was inoculated with 0.2 ml of virus dilution of known titer and incubated at 35°C for 45 minutes. Excess of inoculum was removed and the cells washed once with Hanks' salt solution. Approximately 4.5 ml of melted agar overlay (formula below) were added to each bottle. After agar had solidified, the bottles were turned over and incubated at 35°C for 3-4 days before plaque counts were made. *Agar overlay* was prepared as follows: Melted, sterilized 3% agar (Difco "Noble") in distilled water was mixed with equal portion of nutrient medium consisting of Earle's saline (10 x concentrated without either phenol red or $NaHCO_3$), 18.0 ml; calf serum, 4.5 ml; $NaHCO_3$, 5.4 ml of a 7.5% stock solution; neutral red (1:1000), 3 ml; antibiotics (penicillin 20,000 units/ml, streptomycin 10,000 mu/ml), 2 ml; and distilled water sufficient to make total volume of 90 ml. This mixture then contained 2.25 g of NaHCO/liter. *Virulence test in mice.* For assay of viral virulence, in addition to direct inoculation of TC fluid into mice as previously reported(1), the following procedure was followed: Well separated plaques were harvested with platinum loop, often from those bottles containing only

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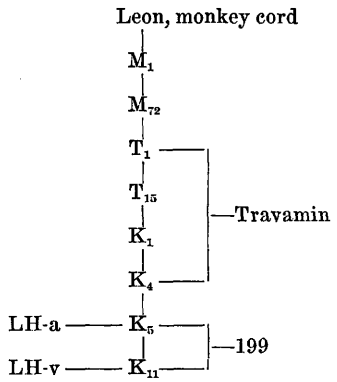

FIG. 1. History of LH-a and LH-v virus.

one plaque. Harvest from each plaque was suspended in 1 ml of Hanks' salt solution, and 0.2 ml of this suspension was inoculated into each of 2 or 3 MKTC tubes, with Hanks' salt solution and 2% calf serum as maintenance medium. This medium was considered as "neutral" because it neither enhanced nor reduced virulence for mice. Then 0.02 ml of pooled TC fluid of tubes from each plaque, (harvested usually on 4th day after inoculation) was inoculated intraspinally into each of 10 or more white mice, 4-5 weeks old. The $TCID_{50}$ of TC fluid usually varied from $10^{6.2}$ to $10^{6.8}$/0.2 ml. Virulence is defined here in terms of percentage of inoculated mice which developed paralysis; virulent, over 50%; avirulent, less than 10% and intermediate, 10-50%.

Results. The mouse-adapted Leon virus was serially passed in mice by intraspinal inoculation and from 72nd passage the virus was inoculated into monkey testicular TC (Fig. 1). All testicular TC and first 4 kidney TC passages were carried out in bovine plasma hydrolysate medium, while the following 7 kidney TC passages were carried out in 199 medium. As reported previously(1,6) the virus lost its mouse virulence after one or 2 passages in monkey testicular TC in plasma clot and bovine plasma hydrolysate medium but regained its virulence after further passages in MKTC in 199 medium. The avirulent passage K_5 was designated as LH-a while the virulent passage K_{11} was designated as LH-v (Fig. 1).

Plaque progeny of LH-v and LH-a virus. The K_{11} virus was plated and 10 plaques were picked. Each plaque was subcultured and the undiluted harvest of subculture of each plaque was titrated in tissue culture and in mice by intraspinal inoculation. Virus dosage/mouse varied from $10^{5.2}$ to $10^{5.8}$ $TCID_{50}$ but this variation seemed to bear little relation to outcome in mice. Among 10 plaques, 7 were virulent, 1 was avirulent and 2 were intermediate. Altogether, 105 mice were inoculated with material from 10 plaques and 63 (or 60%) of them became paralyzed. The avirulent virus of passage K_5 was also plated and subcultures of its 15 progeny plaques were tested in mice. It yielded 9 avirulent, 5 intermediate and 1 virulent plaque. Among 139 mice inoculated with virus from subcultures of these plaques, 10 (or 7%) of them became paralyzed.

Progeny of virulent and avirulent plaques. The question that now arose was whether virulent and avirulent plaques would breed true in a "neutral" medium. One of the virulent plaques from LH-v virus (K_{11}) was picked, subcultured, and replated. Ten progeny plaques were again assayed and they all were virulent. From other experiments an avirulent plaque was picked, and 3 serial plaque passages were made. From each passage 10 plaques were picked, subcultured and assayed in mice and 90% of progeny plaques of each passage were avirulent while the rest were intermediate forms.

Reversion of avirulent variants in Eagle's medium. From virulent LH-v virus (K_{11}), an avirulent culture (K_{12}) was isolated by terminal dilution passage (Fig. 2). This avirulent culture, after further purification twice by terminal dilution and once by plaque method, was again inoculated at its K_{17} passage level into mice as well as plated (Fig. 2). Direct inoculation of TC fluid into mice revealed that the virus was avirulent. By plaque analysis all of its 15 progeny plaques were also avirulent. To see whether this purified avirulent virus could regain its virulence in virulence enhancing media the virus was then serially passed in 2 sets of MKTC tubes, in Eagle's basal medium and 199 medium respectively. After 4 such passages the virus from either set

was still avirulent. However after 7 passages (K_{24}) the virus from Eagle's medium became virulent while that from 199 medium was still avirulent. The viruses from both sets were plated and the one from Eagle's medium yielded 6 virulent out of 10 plaques while the one from 199 medium yielded only avirulent and intermediate plaques (Fig. 2). No effort was made to continue passages in 199 medium which was, as reported previously(1), also a virulence enhancing medium although not as effective as Eagle's medium.

The foregoing experiment, however, did not mean that all avirulent variants could easily regain their virulence in Eagle's medium. In the same experiment, among 15 avirulent plaques yielded by passage K_{17}, plaque $a^{\dagger}$ and $b^{\dagger}$ (Table I) were picked, subcultured and the virus from each of them was serially passed in 2 sets of MKTC, one in Eagle's medium and the other in bovine plasma hydrolysate. After 7 passages, virus from plaque $a^{\dagger}$ regained its virulence in Eagle's medium but not in the other medium, while that from plaque $b^{\dagger}$ did not regain virulence in either medium. (Table I).

The LH-a variant K_5, was also purified, first by terminal dilution 3 times and then

Key for Figures and Tables

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally.

199 = 199 medium. Eagle = Eagle's basal medium. Travamin = Bovine plasma hydrolysate medium.

Te = Terminal dilution passage. Pq = Plaque passage. a, b, c, etc. = No. of individual plaque from which subculture was made for inoculation into mice.

V = Virulent; A = Avirulent; I = Intermediate.

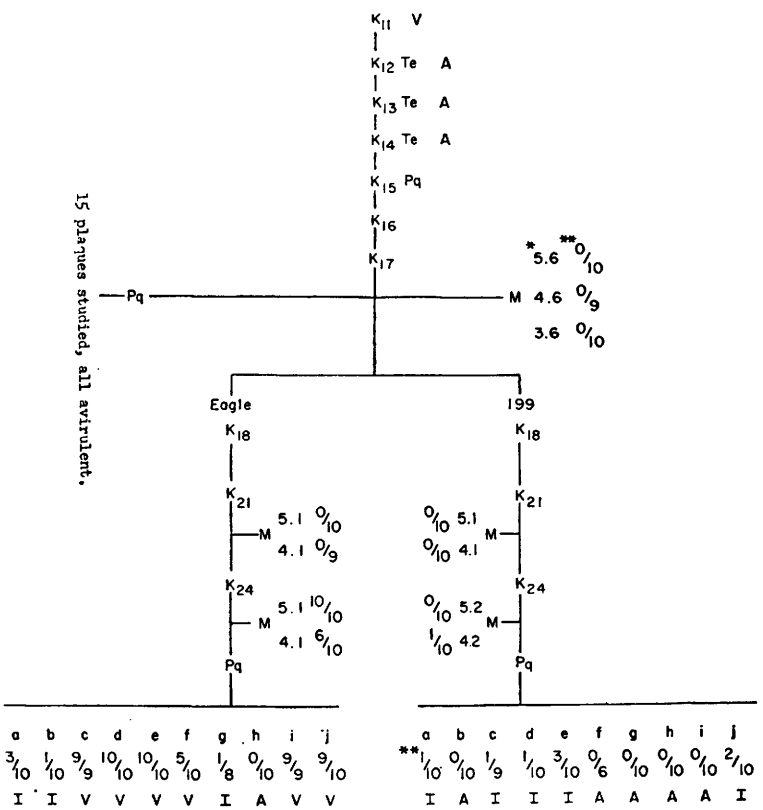

FIG. 2. Reversion of a purified avirulent variant in Eagle's medium.

* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.
** No. of mice paralyzed over No. inoculated.

TABLE I. Reversion of Purified Avirulent Variants in Eagle's Medium.

Derived from	Passage or plaque No.	Virulence before purification	Purified by	No. of passages in Eagle's medium after purification	Result
LH-v, K_{11}	K_{12}	A	Te 3 Pq 1	7	V
	K_{17}, Pq a†	A	Te 3 Pq 2	7	V
	K_{17}, Pq b†	A	Te 3 Pq 2	7	A
LH-a, K_5	K_{11}, Pq c	A	Te 2 Pq 2	16	A
	K_{11}, Pq e	A	Te 2 Pq 2	27	A

once by the plaque method. Subculture from a single plaque was replated and 13 plaques were picked and subcultured. They were all avirulent. Virus from 2 plaques, c and e, was isolated and serially passed, each in two sets, in Eagle's and bovine plasma hydrolysate media, respectively. After 16 and 27 respective passages in Eagle's medium, the virus from both plaques still remained avirulent (Table I), as indicated by direct inoculation into mice at various passage levels and by plating for plaque analysis at end of experiment.

Recovery of virus from inoculated mice. At this point the question was asked: was the failure of the LH-a variant to paralyze mice due to its inability to multiply in the host? During study, spinal cords of some mice inoculated intraspinally with LH-a or LH-v virus were harvested, made into a 10% suspension in saline, centrifuged and supernates were then titrated in MKTC. The results are summarized in Table II. It is rather difficult to measure the extent of increase of virus in spinal cord. In the first place there was no way of knowing how much of the inoculum was absorbed by the spinal cord. Secondly, only the supernate of the cord suspension was used for titration while sediment which contained most of the virus according to our experience, was left out. Nevertheless an approximate comparison could be made. For the LH-v virus the average dosage of inoculum for each mouse was $10^{4.5}$ $TCID_{50}$ and the average $TCID_{50}$ per 0.02 g of the harvested cord tissue was $10^{4.4}$. For the LH-a virus the corresponding figures were $10^{5.5}$ and $10^{2.8}$ respectively.

TABLE II. Recovery of Virus from Spinal Cord of Paralyzed and Non-paralyzed Mice.

Virus inoculated	Mouse No.	$TCID_{50}$ (log 10) inoculated	Days after inoculation	Paralyzed or not	Log $TCID_{50}$ per 0.02 g of spinal cord tissue
LH-v	A	5.0	4	Yes	1.7
	B	5.0	4	"	4.8
	C	5.0	5	"	3.8
	D	5.0	5	"	4.5
	E	5.0	5	"	2.5
	F_1	5.2	5	"	4.3
	F_2	5.0	4	"	5.5
	F_3	5.0	8	"	4.7
	Group 1*	4.3	5	"	4.6
	" 2*	4.3	5	"	4.6
	" 3*	4.3	5	"	4.7
LH-a	A	5.8	4	No	2.7
	B	5.5	4	"	3.0
	C	5.5	5	"	2.5
	D	5.5	5	"	2.7
	F	5.5	6	"	2.2

* 5 mice.

Discussion. These experiments demonstrated that after purification some avirulent virus lines reverted to the virulent phase while others did not. The results suggest that purification by 3 or 4 plaque passages probably did not always yield a homogeneous population, which might explain why in some cases reversion occurred very easily. Or there might be a high back-mutation rate to virulence. It is noteworthy that 2 out of 3 avirulent plaques derived from LH-v reverted to the virulent state while 2 other avirulent plaques derived from LH-a did not revert.

Whether medium 199 in which LH-v had been serially passed was concerned in its genetic instability cannot be answered at present.

Titration in TC of the spinal cords of inoculated mice indicated that LH-a probably multiplied in the host to a lesser degree than LH-v, although both variants multiplied equally well in MKTC. In this respect they behaved like the virulent and attenuated strains of poliovirus in monkeys.

Summary. Both mouse-avirulent LH-a and mouse-virulent LH-v variants of Leon (Type III) poliovirus represented mixed populations, but LH-a yielded more avirulent plaques while LH-v yielded more virulent plaques. On replating, virulent and avirulent plaques usually yielded corresponding virulent and avirulent progeny, although intermediate forms sometimes appeared. After purification, virus from 3 avirulent plaques derived from LH-v was serially passed in Eagle's medium and in 2 of them reversion to virulence occurred; however, in another set of experiments, virus from 2 avirulent plaques derived from LH-a was passed in the same way and reversion did not occur. Titration in TC of suspension of the spinal cords of some inoculated mice indicated that LH-v multiplied more readily in the host than LH-a.

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Experimental Aminonucleoside Nephrosis.(I): Action of Cortisone on Aldosterone and Corticosterone Production. (24035)

D. DAS GUPTA AND C. J. P. GIROUD (Introduced by J. S. L. Browne)
Endocrine Research Laboratory, Montreal Children's Hospital and Department of Investigative Medicine, McGill University, Montreal, Canada

Singer(1) has shown that the nephrotic-like syndrome, induced in rats by an aminonucleoside related to puromycin(2,3) is accompanied by an increased rate of production of aldosterone as measured in the adrenal vein effluent. The purpose of the present work was to confirm this finding and to study the rate of secretion of aldosterone and corticosterone by the adrenals of normal and nephrotic immature rats treated with cortisone acetate.

Methods. Male hooded rats with starting body weight of 75 to 85 g were used. Nephrosis was induced by single daily subcutaneous injections of 6-dimethylaminopurine, 3-amino-d-ribose (1.75 mg in 0.2 ml of water/rat/day for 8 days). Cortisone acetate was given in single daily intramuscular injections for 5 consecutive days, beginning, in nephrotic animals, 24 hours after the last dose of the nephrotoxic drug. Unless otherwise specified, all animals were bled 16-18 hours after being last injected with either the nephrotoxic drug, or cortisone. Adrenal vein blood was collected according to operative procedure of Bush(4) under nembutal anesthesia (7.5 mg/100 g of rat). Since the rate of aldosterone and corticosterone secretion tends to fall markedly with time after one hour of bleeding (personal communication), the period of blood collection was limited to one hour. Blood from 2 to 5 rats was pooled and extracted as described by Bush(4). Residues were submitted to a single paper chromatographic separation in the benzene-aqueous methanol system(5), at room temperature (25 ± 2°C) 5 hours, after overnight equilibration. Corticosterone was eluted with methanol in a modified Haines' device(6), and quantitatively estimated, in duplicate samples, at 2400 Å according to the method of Saffran and Schalley(7). Spectrophotometric meas-