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Studies on Variation in Virulence of Poliomyelitis Virus II. Role of Host Cells and Their Maintenance Medium. (23476)

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In developing strains of avirulent viruses which are satisfactory for immunization, it is important to find out whether there are any environmental factors which can affect the viral virulence. In a previous paper(1) a mouse avirulent variant, (now designated as LH-a), isolated from the mouse adapted Type III poliovirus was reported. On further study, this avirulent variant again yielded a virulent progeny, (designated as LH-v). These 2 variants served as testing tools and with them the effect of various factors on viral virulence was studied and the results are here reported.

Materials and methods. Tissue culture. Primary cultures of trypsinized monkey kidney cells were obtained from a commercial source,† or prepared according to the method described by Dulbecco and Vogt(2) and modified by Youngner(3). These cells were grown in 199 medium with 2% calf serum or in Melnick's medium(4) consisting of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution with 2% calf serum. After growing for about a week, when a complete sheet of cells was formed, the medium was poured off, the cells were washed and fresh medium was added. Such cultures 8 to 15 days old were ready for use in titration, neutralization or passage experiments.

Serial passage cultures of HeLa cells grown in Eagle's basal medium with 20% human serum were supplied commercially.†

All quantitation of the virus in the various harvests was carried out in monkey kidney tissue culture in roller tubes containing lactalbumin hydrolysate medium with 2% calf serum. Serial 1 log or half log dilutions of the virus were made in neutral Hanks' balanced salt solution and 5 or 6 tubes each containing 1.8 ml of medium, were inoculated with 0.2 ml of each of at least 4 dilutions. The tubes were incubated for 7 to 8 days. The tubes were observed microscopically every 2 or 3 days, and the ID_{50} was determined by the Reed and Muench method.

Media. Eagle's basal medium was prepared according to a recently modified formula(5,6). Medium 199 was purchased in

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† Microbiological Associates, Inc., Bethesda, Md.

the concentrated form‡ or prepared by our own media room according to the procedure used by Youngner(3). Lactalbumin hydrolysate medium and Medium E (1 part of bovine plasma hydrolysate and 3 parts of Hank-Simms solution)(7) which contained no serum were prepared in this laboratory. Calf serum used in the preparation of all the media was supplied commercially.§ Every lot of the serum was shown to be free of antibodies or inhibitors against polioviruses before use. For comparison of different media or factors in the same experiment the same lot of serum was used.

Assay of effects of various factors on viral virulence. The effect of various factors including cells and media on virulence of the virus was tested in the following way: Consecutive passage of the testing virus, LH-a or LH-v was carried out in duplicate. Each set contained 5 to 8 tissue culture tubes, including controls without virus inoculation. The same lot of cells was used in each experiment and all the sets were transferred at the same time. From 4 to 10 or more passages were found necessary before loss or gain of virulence of the virus could be clearly demonstrated.

Selected monkey kidney tissue culture tubes were washed twice with the testing media after removal of the original media and then incubated for 24 to 72 hours in the testing media. The testing media were again changed immediately before virus inoculation. All the media were adjusted to the desired pH, 7.4-7.5, by adding diluted sodium bicarbonate solution unless mentioned otherwise. Then 0.2 ml of the undiluted virus in tissue culture fluid was inoculated into each tube containing 1.8 ml of the medium. After virus inoculation the pH of each tube was determined every day. Usually after incubation for 24 hours the pH dropped from 7.4-7.5 to 7.1-7.3 regardless of the medium used. Rarely did the pH drop below 7.0 and then it was brought up to 7.1-7.2.

The tissue culture fluid was harvested when the cellular degeneration became 4+, but never before the 4th day. The harvested virus was then stored and used as inoculum for the next passage. Selected specimens were tested in mice and titrated in tissue culture.

Virus. The LH-a virus as reported previously(1) was derived from the 72nd mouse cord passage of type III poliovirus and passed in the monkey kidney testicular tissue culture in Medium E for 15 passages followed by 4 passages in monkey kidney tissue culture in the same medium. The fluid was centrifuged and filtered through an ultra-fine sintered glass filter and stored in small amounts as stock virus at −50°C. The LH-v virus was prepared and stored in the same way except Medium 199 was used. The specificity of both viruses was repeatedly checked by neutralization tests with type III immune serum obtained from the National Foundation for Infantile Paralysis and also with immune rabbit serum prepared by us against the Saukett strain.

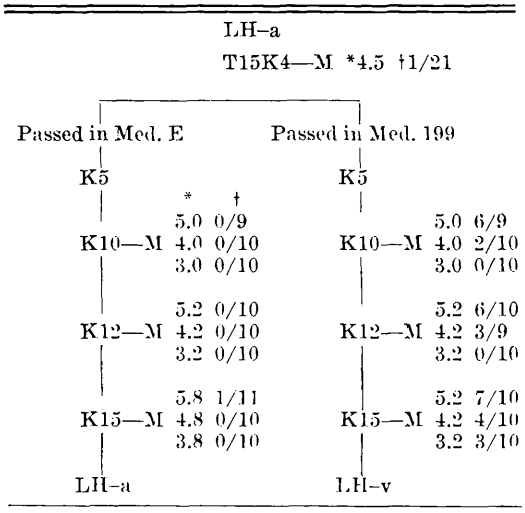
Mice. Assay of viral virulence was made by inoculating 0.02 ml of the undiluted and 10-fold dilutions of the virus in buffered saline solution into 10 to 12 Swiss mice, 4 to 5 weeks old. All inoculations were made intraspinally using a technic described by Habel and Li(8). Inoculated mice were observed for paralysis and death for 2 weeks. The brains and cords of several paralyzed mice were studied histopathologically and poliomyelitis type of lesions was found.

Experimental. During the course of our studies with attenuated poliomyelitis viruses, it was realized that in order to maintain the attenuated character of the variant, it would probably be necessary to maintain the virus under standard culture conditions. However, the striking effect of the medium on the virulence of the LH-a virus was discovered by accident. The mouse avirulent LH-a virus was brought from the virus laboratory in Montgomery to the Nat. Inst. of Health, Washington, D.C. in 1955. The virus was then serially passed in tissue culture and inoculated intraspinally into mice. Unexpectedly, some of the mice became paralyzed. At first it was thought that this effect was due to the difference in the strains of the mice used but the

‡ Parke, Davis and Co., Detroit, Mich.

§ Merck, Sharpe & Dohme, Philadelphia, Pa.

CHART 1. Effect of Maintenance Medium of Monkey Kidney Tissue Culture on Mouse Virulence of LH–a Virus.



Key:

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

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

† No. of mice paralyzed over No. inoculated.

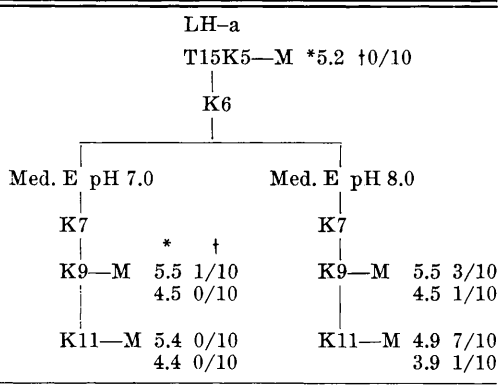
assumption turned out to be incorrect. The other possible explanation was that Medium E was used for tissue culture in Montgomery while Medium 199 was used in Washington. In order to test the effect of the medium on the virulence of the virus, Exp. I was set up which led to a number of experiments, a few examples of which are described below:

Exp. I. Comparison of Medium E and Medium 199. The LH-a virus, T15K4 passage was inoculated into 2 sets of monkey kidney tissue culture tubes. In one set Medium E was used while in the other, 199 medium with 2% calf serum was used. Both media were adjusted to the same pH, 7.4-7.5. Comparable tissue culture fluids of both sets were titrated in mice and in tissue culture. As shown in Chart I the virus from the Medium E set was avirulent for mice, even $10^{5.2}$ $TCID_{50}$ virus (K12) did not paralyze the inoculated mice. Virus from Medium 199, however, was virulent for mice; even $10^{3.2}$ $TCID_{50}$ virus (K15) paralyzed some of the inoculated mice. This mouse virulent variant was designated as LH-v and was used as a testing virus for further experiments. The passages were carried further than that indicated in Chart I and were also repeated many times and similar results were obtained, except that there was slight variation in the effect of 199 media from different sources. It was also shown by further experiments that the presence or absence of calf serum in the medium made no significant difference.

Exp. II. Effect of the pH of medium. Dulbecco, Vogt and Sabin(9) discovered a distinct relationship between neurotropism for the monkey and plating efficiency of poliovirus. We could confirm the effect of the pH of the medium on virulence of the virus but the effect was not in the same direction as shown by these authors, possibly because we were testing virulence in mice rather than in monkeys. One of the experiments along this line was carried out as follows: The virus LH-a, T15K5 passage was inoculated into 2 sets of monkey kidney tissue culture tubes. Medium E was used for both sets but in one set the pH was adjusted to 7.0, in the other to 8.0. Both sets were transferred for 5 passages and the tissue culture fluid from the third (K9) and the fifth (K11) passage were titrated in tissue culture and in mice. The virus from the set with a high pH (8.0) was virulent for mice while the virus from the other set was avirulent (Chart 2). Obviously, in order to test the effect of a given medium in comparison with another, the pH of both media must be kept constant.

Exp. III. Comparison of Eagle's, 199 and Lactalbumin Media. In this experiment the LH-a virus, T15K5, was inoculated into 3 sets of monkey kidney tissue cultures fed with lactalbumin hydrolysate medium, 199 medium, and Eagle's basal medium respectively. The purpose of this experiment was to find out whether there were any media other than Medium 199 which would restore the virulence of the virus. As shown in Chart 3, in the Eagle's basal medium, the virus became highly virulent after 4 passages. In the 199 medium it took 6 passages for the virus to become virulent. In the lactalbumin hydrolysate medium the virus remained avirulent after 6 passages. This experiment demon-

CHART 2. Effect of pH of Medium of Tissue Culture on Mouse Virulence of LH–a Virus.



Key:

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

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

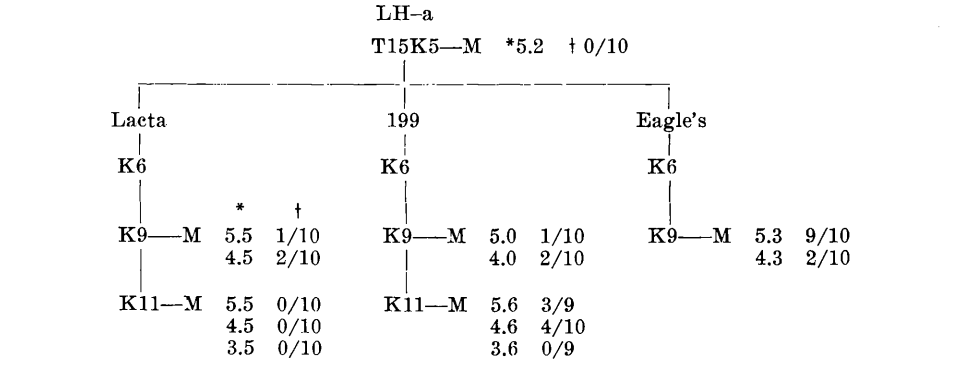
† No. of mice paralyzed over No. inoculated.

strated that both the Eagle basal medium and 199 medium belonged to the virulence enhancing group but the former was more effective. The lactalbumin medium behaved like Medium E.

Exp. IV. Reversibility of LH-v Virus. In the foregoing experiments, it was demonstrated that the virulence of the LH-a virus could be restored by passages in monkey kidney tissue culture fed with Medium 199 or Eagle's basal medium, yielding the mouse virulent variant LH-v. An experiment was conducted to ascertain whether the virulence of the LH-v virus could again be reduced or nullified by changing the medium. The LH-v virus, taken from T15K14 passage in 199 medium (Chart 1) was inoculated into 3 sets of monkey kidney tissue culture tubes overlaid with Medium E, Medium 199 and lactalbumin medium respectively, (Chart 4). Five serial passages were carried out and comparable fluids from each group were titrated in tissue culture and in mice. As shown in Chart 4, the virus became avirulent after 5 passages in Medium E and lactalbumin medium but remained virulent in Medium 199.

Exp. V. Comparison of different cells in the same medium. It was shown previously(1) that the change in virulence was related to the kind of host cells in which the virus was propagated. This relationship was confirmed in the present study and an illustrative example is given below: The LH-v virus from K15 passage (Chart 1) was inoculated into 3 sets of tissue culture tubes. In Set 1, HeLa cells were used, while in Set 2 monkey kidney cells were used, but in both sets the cells were overlaid with the same 199 medium with 2% calf serum. In Set 3, the same monkey kidney cells were used but they were overlaid with Medium E. All the media were adjusted to pH 7.5. Five serial passages in the respective

CHART 3. Comparison of Effect of Eagle's Basal Medium, 199 Medium and Lactalbumin Medium of Tissue Culture on Mouse Virulence of LH–a Virus.



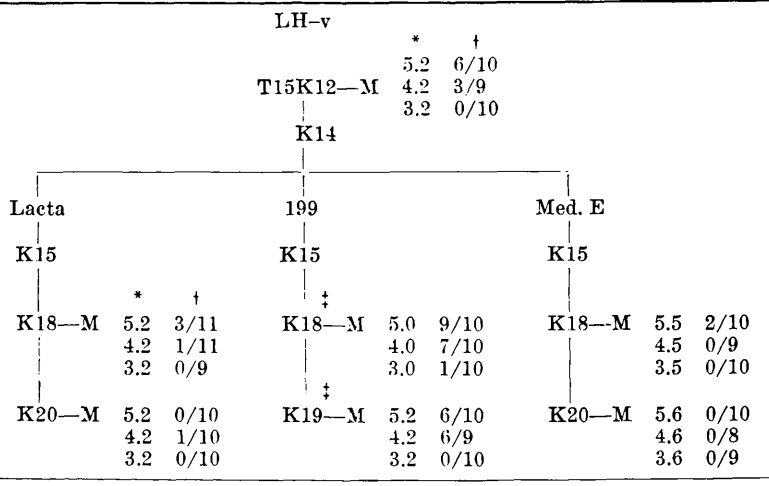
Key:

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally. Lacta = Lactalbumin medium. 199 = 199 medium. Eagle's = Eagle's basal medium.

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

† No. of mice paralyzed over No. inoculated.

CHART 4. Reversibility of Mouse Virulence of LH–v Virus Passed in Tissue Culture.



Key:

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally. Lacta = Lactalbumin medium. 199 = 199 medium.

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

† No. of mice paralyzed over No. inoculated.

‡ K19 was not as virulent as K10 as a different preparation of 199 medium from a different source was used for K19.

media were carried out, and the harvests from the last 2 passages of each set were titrated in tissue culture and in mice.

In the same host cell (monkey kidney) the virus lost its virulence in Medium E but retained its virulence in Medium 199 (Chart 5). This was the usual finding, as shown in Exp. 4. The striking result was that the virus lost its virulence completely in the HeLa cell culture but retained its virulence in monkey kidney cell culture although the cells in both sets were overlaid with the same medium at the same pH.

Discussion. In studying the effect of various factors on the mouse virulence of the type III poliovirus, it was advantageous to have a strain of readily variable virus so that any slight effect of the testing material on the virulence could be demonstrated. It was also necessary to have two variants; one mouse virulent for testing virulence reducing factors and the other mouse avirulent for testing virulence enhancing factors. The availability of both of these variants (LH-a and LH-v) made this study possible. However, neither variant had been purified, *i.e.*, both represented mixed populations of virulent and avirulent particles, but in the LH-a variant there were more avirulent particles than virulent particles and the reverse was true for the LH-v variant. Further work indicated that purified variants isolated by terminal dilution and the plaque method were less readily variable so they were not good testing tools. On the other hand they could still be made virulent or avirulent by the same measures used in the present study except that it took more serial passages to accomplish the task (10).

In this connection it must be mentioned that continuous passages of the LH-a variant in the virulence reducing system seemed to stabilize the avirulent character of this variant for it took many more passages in a virulence enhancing medium to restore its virulence. Continuous passages of the LH-v variant in the virulence enhancing medium would also make it more virulent and less readily reversible, (*e.g.*, in Medium 199, K15 was more virulent than K12, as shown in Chart 1).

Summary. A method of testing the effect of various factors on the mouse virulence of the type III poliovirus was described. Variants of the virus which could be made virulent or

CHART 5. Comparison of Mouse Virulence of LH–v Virus Passed in Different Host Cells Maintained in Medium 199.

		*	†
LH–v T15K12 K13	K15—M	5.2	7/10
		4.2	4/10
		3.2	3/10
199 H1	H5—M	5.5	0/7
		4.5	0/10
		3.5	0/9
	H6—M	5.4	0/10
		4.4	0/5
		3.4	0/10
199 K16	K19—M	5.5	8/10
		4.5	4/10
		3.5	0/10
	K20—M	5.0	8/10
		4.0	3/11
		3.0	0/11
Med. E K16	K19—M	5.5	0/10
		4.5	1/10
		3.5	0/10
	K20—M	5.0	2/10
		4.0	0/11
		3.0	0/10

Key:
T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. H = HeLa cell tissue culture passage. M = Mice inoculated intraspinally. 199 = 199 medium.
* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.
† No. of mice paralyzed over No. inoculated.

avirulent were isolated. The virus was designated as LH-v in its virulent phase and LH-a in its avirulent phase. Using these variants as testing tools, 3 factors affecting the viral virulence were found; namely, the host cell, the maintenance medium, and the pH of the medium. These factors were classified into 2 categories, namely the virulence reducing group and the virulence enhancing group. When tested with monkey kidney cell cultures in the pH range 7.0-7.5, Eagle's basal medium and 199 medium belonged to the virulence enhancing group while Medium E (bovine plasma hydrolysate) and lactalbumin hydrolysate medium belonged to the virulence reducing group. When tested in Medium 199 at pH 7.0-7.5 monkey kidney cells enhanced the viral virulence while HeLa cells behaved in the opposite way.

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