6. Li, C. P., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 233.

7. Moyer, A. W., Accorti, C., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 513.

8. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.*, 1951, v94, 111.

9. Casals, J., and Olitsky, P. K., *Arch. f. ges. Virusforsch.*, 1951, v4, 452.

10. Selzer, G., Sacks, M., and van den Ende, M., *South African Med. J.*, 1952, v26, 201.

11. Paul, J. R., Poliomyelitis. Papers and discussions presented at the First International Poliomyelitis Conference, J. B. Lippincott, Philadelphia, 1949, p271.

12. Casals, J., and Olitsky, P. K., *Diagnosis of Viral and Rickettsial Infections,* 1949, Chap. 6, p71. F. L. Horsfall, Jr., Editor, Columbia University Press, New York.

13. Burnet, F. M., *Med. J. Australia,* 1935, v1, 46.

14. Stimpert, F. D., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 483.

15. Kast, C., and Kolmer, J. A., *J. Infect. Dis.,* 1937, v61, 60.

16. Gard, S., *Nature,* 1943, v152, 660.

17. Schultz, E. W., and Enright, J. B., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 8.

18. Enright, J. B., and Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 541.

19. Warren, J., *Viral and Rickettsial Infections of Man,* 1952, Chap. 39, p. 675, Thomas M. Rivers, Editor, J. B. Lippincott Co., Philadelphia.

20. Gard, S., *Acta Med. Scand.,* (Suppl. 143), 1943, v143, 173.

21. Dunham, W. B., and Parker, S., *J. Bact.,* 1943, v45, 80.

22. Enders, J. F., *Viral and Rickettsial Infections of Man,* 1952, Chap. 6, p134, Thomas M. Rivers, Editor, J. B. Lippincott Co., Philadelphia.

23. Koprowski, H., Norton, T. W., and McDermott, W., *Public Health Rep.,* 1947, v62, 1467.

24. Schlesinger, R. W., *Am. J. Hyg.,* 1950, v51, 248.

Received October 9, 1952. P.S.E.B.M., 1952, v81.

Poliomyelitis. III. Propagation of MEF1 Strain of Poliomyelitis Virus in Developing Chick Embryo by Allantoic Cavity Inoculation.* (19929)

VICTOR J. CABASSO, MAX R. STEBBINS, RAY M. DUTCHER, ARDEN W. MOYER, AND HERALD R. COX.

From Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Co. Pearl River, N. Y.

The numerous unsuccessful attempts to propagate poliomyelitis virus in the developing chick embryo have led investigators to consider this viral agent as being uncultivable in this experimental host and to use this characteristic as a distinguishing feature between poliomyelitis and other neurotropic agents(1). Similar unsuccessful results had repeatedly been obtained with viruses other than poliomyelitis which later, however, were successfully grown in the chick embryo. Such, for example, was the case with canine distemper virus which was propagated in fertile hen's eggs almost simultaneously by Haig(2) in South Africa and by two of us(3) in this country. Equally successful efforts are now being reported with a Lansing type strain of poliomyelitis virus. In the two preceding papers of this series, Moyer and coworkers (4) have described the serial passage of the MEF1 strain of poliomyelitis virus in the suckling hamster with a resulting increase of its LD_{50} titer for mice, and Roca-Garcia and coworkers(5) have reviewed the pertinent literature and reported the propagation of Moyer's MEF1-A strain in the developing chick embryo by the yolk sac route of inoculation. The present communication describes the propagation of Moyer's hamster-adapted virus by inoculation into the allantoic sac of the developing chick embryo. The experimental work reported here was conducted independently, with different personnel and in separate quarters.

Materials and methods. Virus strains. The

* The authors wish to express their gratitude to Mrs. Violet Pedersen for her able technical assistance, and to Dr. Floyd S. Markham and Mrs. Agnes K. Lamb for their suggestions in the preparation of the manuscript.

strain employed in this study was the MEF1 Lansing type of poliomyelitis virus, which has been serially passed in unweaned hamsters by one of us(4) and designated as MEF1-A. The preparations used for egg inoculation were 20% suspensions of baby hamster brain representing the 131st serial hamster passage which had a mouse LD_{50} titer of $10^{6.52}$. The MEF1 strain(6) which was adapted to suckling mice (7), and for which we are indebted to Dr. Jordi Casals of the Rockefeller Foundation Laboratories, New York, N. Y., was used in the cross-neutralization tests on monkey sera.

Chick embryos and passage method. The chick embryos employed were 7 days old when inoculated in the allantoic cavity with 0.25 ml of either infected hamster brain or chick embryo tissue suspensions. Inoculated eggs were incubated at 37°C first for 7 days and in later egg passages for 4 to 5 days. Twenty per cent tissue suspensions were routinely employed for passage. *Mice.* Three-week-old Swiss albino mice weighing 10 to 12 g each were used for titration and standard neutralization tests.

Immune sera. The immune and normal sera used for the identification of the chick embryo propagated virus were derived from either hamsters or monkeys. The following sera were inactivated in the water bath at 56°C for 30 minutes before their use in this study: 1) an immune hamster serum derived from animals hyperimmunized with the hamster propagated MEF1-A virus at its 86th passage in hamsters; 2) immune monkey sera against either Brunhilde, Leon or Lansing A types of virus, received through the courtesy of Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md., to whom we are very grateful; 3) immune monkey sera against either Brunhilde, Leon or Y-SK Lansing type virus, very kindly sent to us by Dr. Joseph F. Melnick, Yale University, New Haven, Conn.; and 4) a normal hamster serum which was used as control.

Neutralization tests. Identification of the virus adapted to chick embryo passage was based on standard neutralization tests. Equal volumes of undiluted serum and serial tenfold dilutions of infected chick embryo suspensions were mixed, incubated in a water bath for one hour at 37°C and inoculated into mice by the intracerebral route. The mice were observed for 21 days and the LD_{50} titers were calculated according to the Reed and Muench formula(8). Neutralization indices were derived from the differences between the log LD_{50} titers of normal and immune serum mixtures.

Experimental. Adaptation of the virus to the developing chick embryo. Of the numerous attempts made in this laboratory to propagate poliomyelitis virus in the chick embryo by using different routes of inoculation, different incubation temperatures and embryos of different ages, the following series was successfully carried through at least 40 serial chick embryo passages: On April 15, 1952, a 20% suspension of suckling hamster brain representing the 131st serial passage of the MEF1-A virus(4) was injected in the allantoic cavity of 8, 7-day-old embryos, each embryo receiving 0.25 ml volume. These eggs, which constituted the first chick embryo passage of the present series, were incubated at 37°C for 7 days. At the end of this incubation period embryos and chorio-allantoic membranes of inoculated eggs were harvested aseptically, pooled, ground in a Waring blendor and centrifuged at 2,000 rpm for 20 minutes in a size 1 International anglehead centrifuge. The undiluted suspension was immediately titrated in tenfold dilutions in mice. Four mice were used for each dilution and each mouse received 0.03 ml intracerebrally of the respective dilution. A 20% suspension was in turn made from the undiluted material of the first chick embryo passage and inoculated via the allantoic cavity into 8, 7-day-old chick embryos and these also were incubated at 37°C for 7 days. Harvesting and mouse titration of material from this second passage and from the 8 succeeding passages were made in a manner identical to that used for the first passage.

Meanwhile, having found by mouse titrations that a 4- to 5-day incubation period at 37°C yielded higher concentrations of virus in the embryonic tissues than did a 7-day incubation period, the shorter time interval was used with chick embryo passages higher

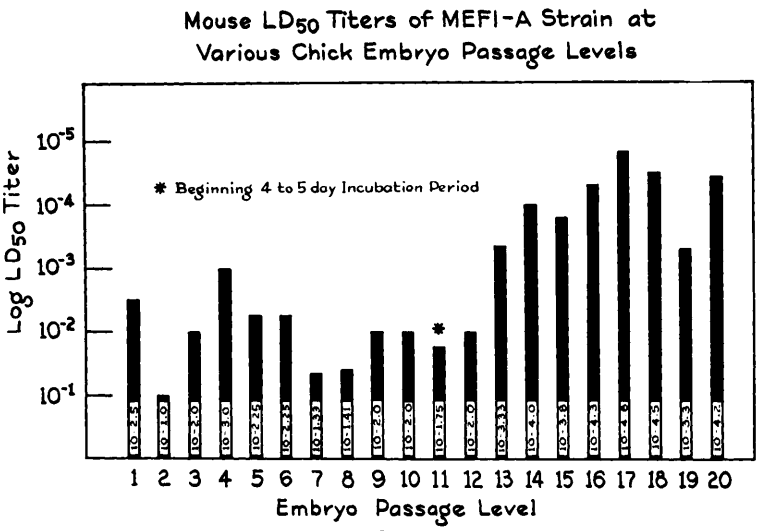

FIG. 1.

than the 10th. Methods of harvest, mouse titration and egg inoculation, however, remained the same as those used in earlier passages. At the time of writing, 40 successive passages of this virus in chick embryos have been made. It may be noted in the accompanying chart that beginning with the 13th passage in chick embryos the mouse LD_{50} titers increased, reaching a maximum of $10^{-4.8}$ at the 17th chick embryo passage level.

Identity of the passage virus. Virus tissue suspensions representing the 6th, 13th and 16th chick embryo passages were employed in standard neutralization tests. The sera used in these tests were: 1) an immune hamster serum against the parent MEF1-A strain of virus; 2) immune monkey sera against the homotypic Armstrong and Y-SK strains of Lansing virus; and 3) immune monkey sera against the heterotypic Brunhilde and Leon virus strains. The results of the neutralization tests are summarized in the accompanying table. The data indicate that the passage virus propagated serially by the allantoic cavity route in the developing chick embryo is a *bona fide* strain of Lansing type poliomyelitis virus. The passage virus was not only neutralized by an immune hamster serum prepared against the MEF1-A strain of Moyer, but it was also neutralized by immune monkey sera prepared in other laboratories against homotypic Lansing type strains. A slight degree of neutralization may be observed also in the tests of the 13th and 16th passage level virus in the presence of one of the Brunhilde and both of the Leon immune monkey sera. It is difficult to assess the significance of this slight heterotypic neutralization, but in light of the recent observations of Casals *et al.*(9,10) and Sabin(11), it is suggestive of antigenic overlapping within the group of poliomyelitis viruses.

Virulence tests in monkeys and chimpanzees. A 50% chick embryo tissue suspension having a mouse LD_{50} titer of $10^{-3.3}$ was prepared from the 13th passage and used in a preliminary test of virulence in Rhesus monkeys. Two monkeys (2441 and 2442) each received 0.5 ml intracerebrally, and two others (2443 and 2444) were injected intramuscularly with 3.0 ml. One of the intracerebrally injected monkeys (2442) developed weakness of the hind legs and tremor beginning on the 9th day after inoculation, but recovered completely 9 days later. Monkey 2444 died of tuberculosis 19 days after intramuscular injection. The other two monkeys remained free of nervous symptoms. Serum samples obtained from the 3 surviving monkeys 35 days after injection were tested for antibodies by mouse neutralization tests. Against Casals' suckling mouse propagated MEF1 strain of virus(7),

TABLE I. Results of Serum-Neutralization Tests in Mice with Chick Embryo Adapted MEF1-A Virus and Immune Sera Against Lansing, Brunhilde and Leon Types Viruses.

Egg passage level	Results	Serum-virus mixtures: Lansing immune sera: MEF1 hamster	Lansing immune sera: Lansing A monkey*	Lansing immune sera: Y-SK monkey†	Brunhilde immune sera: Monkey*	Brunhilde immune sera: Monkey†	Leon immune sera: Monkey*	Leon immune sera: Monkey†	Normal control: Hamster
6	LD_{50} titer	$<10^{-1}$	—	—	—	—	—	—	$>10^{-4.5}$
	N‡	>3162							
13	LD_{50} titer	$<10^{-1}$	$<10^{-1}$	—	$10^{-2.5}$	—	$10^{-3.3}$	—	$10^{-4.7}$
	N‡	>5012	>5012		158		25		
16	LD_{50} titer	$10^{-2.1}$	—	$10^{-1.6}$	—	10^{-6}	—	10^{-4}	$10^{-5.5}$
	N‡	2512		7943		0		32	

* Supplied by Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md.

† Supplied by Dr. Joseph F. Melnick, Yale University, New Haven, Conn.

‡ N = Neutralization index.

the serum of one of the intracerebrally injected monkeys (2442) had a neutralization index of 316, while that of its mate (2441) was only 21. The serum of the surviving intramuscularly injected monkey (2443) had an index of only 25. The complement-fixing titers of these 3 sera were found to parallel their neutralization indices. Thus, monkey 2442 showed a complement-fixing titer of 4+ at 1:16 serum dilution; monkey 2441 showed 3+ at 1:2 dilution; and monkey 2443 failed to give a significant complement-fixing response.

A 20% chick embryo tissue suspension of the 17th passage having a mouse LD_{50} titer of $10^{-4.8}$ was titrated intracerebrally in duplicate monkeys. A dose of 0.5 ml of the undiluted suspension, or of 10^{-1}, 10^{-2} or 10^{-3} dilutions thereof, was injected. During the 21-day observation period none of the 8 monkeys showed paralytic or other nervous symptoms. Serum samples from these animals are to be assayed for neutralizing antibodies and the monkeys are to be tested for active immunity by intracerebral challenge. The same virus-tissue suspension was fed to 2 chimpanzees and injected intramuscularly into 2 additional chimpanzees in an attempt to determine its pathogenicity and antigenicity by the oral or intramuscular routes. All 4 chimpanzees have remained normal to date, at least 3 months after exposure. Results of the neutralization tests performed in mice with the 14th, 28th, 42nd and 56th day serum samples of the 4 chimpanzees against both the parent MEF1 and the Y-SK strains of virus, indicate the presence of neutralizing antibodies in the serum of all 4 animals against both virus strains. Details of the final results will be reported in a later communication.

Adaptation of the MEF1-B Moyer strain to the chick embryo. Using the technic described above, the MEF1-B Moyer strain of suckling hamster virus was also adapted to grow in the developing chick embryo and, at the time of writing, is in its 23rd egg passage. Mouse LD_{50} titers increased from $10^{-4.0}$ at the first egg passage level to greater than $10^{-7.0}$ at the 7th passage. A neutralization test in mice with the 6th chick embryo passage virus and the homologous immune hamster serum clearly shows the identity of this virus with the Lansing type poliomyelitis strain.

Discussion. Because of the many unsuccessful attempts made to cultivate poliomyelitis virus in the developing chick embryo, most investigators were led to believe that this group of agents was not able to parasitize this host and that this characteristic could be used to distinguish poliomyelitis virus from other neurotropic agents(1). One of us(12), however, expressed his confidence that someone would eventually succeed in adapting poliomyelitis virus to the chick embryo. As reported above, the present authors have been successful in serially propagating both of Moyer's(4) MEF1-A and MEF1-B hamster passaged virus strains in the chick embryo, with significant increases of their mouse LD_{50} titers in the higher serial embryo passages.

Whether this adaptation was made possible by the increased titers obtained in the serial hamster passages is difficult to determine at this time, but this point is being investigated. The adaptation to the chick embryo of this Lansing type poliomyelitis virus offers new promise both in the field of diagnosis as well as of prophylaxis against the disease. Tests already carried out in monkeys strongly indicate that the chick embryo-propagated virus has lost in great measure its virulence for primates, inducing no severe disease symptoms even when administered intracerebrally in comparatively massive doses. Furthermore, accumulating evidence obtained in this laboratory as well as by other workers(9-11) shows a certain antigenic overlapping between the Lansing type and the Brunhilde and Leon types of poliomyelitis agents. This gives reason to hope that immunity engendered by one type of virus may also elicit an adequate basic immunity against heterotypic strains whereby some of the difficulties encountered in diseases with a plurality of strains may be overcome. It is also evident that the use of the developing chick embryo as a source of virus infected tissues may obviate many of the dangers and difficulties encountered in the use of infected rodent brain suspensions or tissue culture preparations requiring mammalian tissues.

The oral administration of a living, modified virus, such as one described here, seems to offer the most logical approach as a prophylactic measure against poliomyelitis(13). Whether a single oral dose would be sufficient for the adequate production of both homo- and heterotypic antibodies will have to be determined by further experimental work which is being carried out.

Summary and conclusions. Data describing the cultivation of the MEF1 Lansing type poliomyelitis virus in the developing chick embryo are reported. This strain of poliomyelitis virus was derived from the original MEF1 strain following its adaptation to suckling hamsters. Neutralization tests performed at three passage levels with *bona fide* immune hamster and monkey sera prepared against three homotypic strains of the Lansing type virus point to the identity of the chick embryo adapted and propagated virus with this type of poliomyelitis agents. A moderate neutralization has also been shown to take place between the chick embryo adapted virus and immune monkey Brunhilde and Leon sera, but, as indicated, it is difficult to attribute much significance to this finding at the present time. Some experimental evidence was obtained indicating that the chick embryo adapted virus does not induce paralysis or death in Rhesus monkeys even following intracerebral inoculation with comparatively massive doses of virus. The effect of this virus on the immune response of Rhesus monkeys and chimpanzees is being further investigated.

1. Casals, J., and Olitsky, P. K., *Diagnosis of Viral and Rickettsial Infections,* F. L. Horsfall, Jr., Editor, Columbia University Press, New York, 1949, p71.

2. Haig, D. A., *Onderstepoort J. Vet. Sci. and An. Ind.,* 1948, v23, 149.

3. Cabasso, V., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 246.

4. Moyer, A. W., Accorti, C., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1952, (this issue).

5. Roca-Garcia, M., Moyer, A. W., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1952 (this issue).

6. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science,* 1943, v98, 452.

7. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.,* 1951, v94, 111.

8. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

9. Casals, J., Olitsky, P. K., and Sabin, A. B., *J. Exp. Med.,* 1952, v96, 35.

10. Casals, J., Olitsky, P. K., and Brown, L. V., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 731.

11. Sabin, A. B., *J. Exp. Med.,* 1952, v96, 99.

12. Cox, H. R., *Annals N. Y. Acad. Sci.,* 1952, v55, 236.

13. Koprowski, H., Jervis, G. A., and Norton, T. W., *Am. J. Hyg.,* 1952, v55, 108.

Received October 9, 1952. P.S.E.B.M., 1952, v81.