Poliomyelitis. II. Propagation of MEF1 Strain of Poliomyelitis Virus in Developing Chick Embryo by Yolk Sac Inoculation.* (19928)

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Since Armstrong's discovery in 1939(1) that a strain of poliomyelitis virus, now known as Lansing or Type 2 virus, may be transmitted to cotton rats, several investigators have been successful in adapting other Lansing type strains(2-5) and a Leon, Type 3 virus(6) to rodents. Recently, our laboratory(7) has succeeded in serially infecting suckling hamsters with the MEF1 strain Lansing type poliomyelitis virus by the intracerebral route, thereby bringing about a considerable increase of its LD_{50} titer for mice. While our work was in progress, Casals *et al.*(8,9) and Selzer and coworkers(10) reported the adaptation of the MEF1 strain Lansing type virus to newborn mice. Although the increase of its LD_{50} titer was not of the same magnitude as that obtained in suckling hamsters(7), the newborn mouse-adapted virus has yielded a diagnostic antigen for the complement-fixation test. The more ready transmissibility of the Lansing type strains of virus to rodents has been used to differentiate this group of viral agents from those of the Brunhilde or Leon types(11). Furthermore, the inability of any of the known types or strains of human poliomyelitis virus to grow in the developing chick embryo has been considered a characteristic distinguishing these viruses from other neurotropic agents(12). Of the several fruitless attempts made to adapt human poliomyelitis viruses to the developing chick embryo, those of Burnet(13), Stimpert(14) and Kast and Kolmer(15) may be cited. Gard(16) was able to infect a monkey by injecting it with the brain tissue of chick embryos inoculated ten days previously with a strain of poliomyelitis virus, but obtained no evidence of true virus multiplication. Schultz and Enright(17,18) reported the cultivation in the chick embryo of the SK, MM and C(M) strains of so-called "murine poliomyelitis" virus, now recognized as strains of encephalomyocarditis virus(19). Schultz and Enright also adapted Theiler's GDVII strain of mouse encephalomyelitis virus to the chick embryo, thus confirming the work of Gard, Dunham and Parker(20,21). They failed, however, to propagate 5 strains of human poliomyelitis virus in the chick embryo. More recently, Enders(22) and his associates reported failure to propagate a Lansing type strain of poliomyelitis virus in chick, beef or mouse embryonic tissue or in the testicular tissue of the rabbit, whereas the agent was readily propagated in cultures of human or monkey tissues.

The purpose of this communication is to present experimental evidence of the propagation of the MEF1 strain of poliomyelitis virus by the yolk sac route of inoculation in the developing chick embryo, following its adaptation to suckling hamsters.

Materials and methods. Virus strains: The virus used to initiate the 2 embryo passage series described below represented the 119th hamster passage of the MEF1-A strain of Moyer *et al.*(10). In the mouse neutralization tests either the 64th or 131st hamster passage of Moyer's MEF1-A strain was employed for the determination of antibodies in the experimental monkey sera. The challenge virus used to test the immunity of monkeys was a pooled monkey cord suspension prepared from the 4th monkey passage of the Armstrong Lansing strain, which was obtained in its 197th mouse passage through the courtesy of Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md. This virus is referred to in the text as pooled monkey cord Lansing virus.

Chick embryos and passage method: Unless otherwise stated, 7-day-old chick embryos were incubated for 7 or 8 days at 37°C after yolk sac inoculation with an 0.6 ml dose of

* The authors wish to express their gratitude to Mrs. Vivian Scully, whose loyal and conscientious efforts helped make this work successful; and to Mrs. Agnes K. Lamb and Dr. Floyd S. Markham for their helpful suggestions in the preparation of the manuscript.

a 66% tissue suspension. Embryos were harvested and homogenized in a Waring blendor with 3 ml of buffered saline per embryo, giving an approximate 66% tissue suspension, referred to hereafter as undiluted material. The resulting tissue suspension was centrifuged for 10 minutes at 1500 rpm in a No. 1 International angle head centrifuge. The supernate was used for chick embryo passage and for the inoculation of test animals only when found to be bacteriologically sterile.

Immune sera: A Lansing type immune serum prepared in our laboratory by hyperimmunization of a monkey which survived intracerebral inoculation with the WW strain of virus(23) was commonly used. This Lederle serum has been employed routinely as a standard Lansing type immune serum and its specificity has been repeatedly demonstrated. In addition, for critical neutralization tests, a Y-SK immune monkey serum was obtained through the courtesy of Dr. Joseph L. Melnick of Yale University, New Haven, Conn., and a Lansing (Armstrong) immune serum was received from Dr. Howe. A GDVII immune serum prepared in rabbits immunized by 25 weekly injections of GDVII infected mouse brain suspensions was also employed.

Neutralization tests: The neutralization test procedure was carried out as follows: Undiluted normal and immune sera, respectively, were mixed with serial dilutions of the virus under test. The mixtures were incubated at 37°C for 90 minutes before intracerebral injection in mice. Neutralization indices were calculated on the basis of the difference between the log LD_{50} titers obtained with normal and immune serum, and are expressed as the number of LD_{50} doses of virus neutralized by undiluted serum.

Test animals: Swiss albino mice 28 to 35 days old were used for virus titrations and serum neutralization tests. Rhesus monkeys (*Macaca mulatta*) weighing 7 to 8 pounds were employed for virulence tests and virus titrations.

Experimental. Chick embryo series R-302. This series was initiated in chick embryos on February 4, 1952, with a 10% cord suspension prepared from the second mouse brain passage of the 119th hamster passage of Moyer's MEF1-A Lansing type poliomyelitis virus. Live embryos were harvested 4 days later and a 66% suspension prepared and injected intracerebrally at once into 3 Swiss albino mice. Two days later a second group of 2 mice were similarly inoculated with the remainder of the chick embryo suspension, which had been stored in the meantime at 4°C. All 5 mice of the 2 groups became paralyzed 4 to 6 days after inoculation. A 10% suspension was prepared from the pooled cord tissues of the paralyzed mice of these 2 groups, and chick embryos were inoculated by the yolk sac route with the cord suspension. The embryos were harvested on the 7th day, homogenized and inoculated into 4 additional mice. Separate brain and spinal cord suspensions were prepared from the 4 mice, all of which became paralyzed. Separate serial egg passages labeled R-302-cord (R-302-C) and R-302-Brain (R-302-B) were made from the indicated mouse tissues on March 3, 1952. Live embryos were harvested from each egg passage on the 4th and again on the 7th day after inoculation. Pending infectivity tests in mice, the remaining portions of the harvested chick embryo tissue suspensions were shell frozen and held at —30°C for 7 days. The mouse tests demonstrated the presence of virus in embryos harvested at 7 days both in egg passages R-302-C and R-302-B. The frozen reserve suspensions from these two, first chick embryo passages were therefore pooled to initiate the second chick embryo passage R-302-2 in 26, 7-day-old chick embryos. Thereafter, this strain (R-302) has been maintained in serial uninterrupted chick embryo passages for 41 consecutive generations. Groups of 4 to 7 mice were injected intracerebrally with undiluted chick embryo suspensions at each passage level. The inoculated mice invariably developed paralysis and died. Tissue suspensions from the 8th, 9th, 11th and 13th and all subsequent chick embryo passages were titrated intracerebrally in mice. The results of these titrations are presented in the accompanying chart.

Neutralization tests for identification of the passage virus were performed in mice with chick embryo tissue suspensions, or with brain and cord tissue suspensions of mice which had

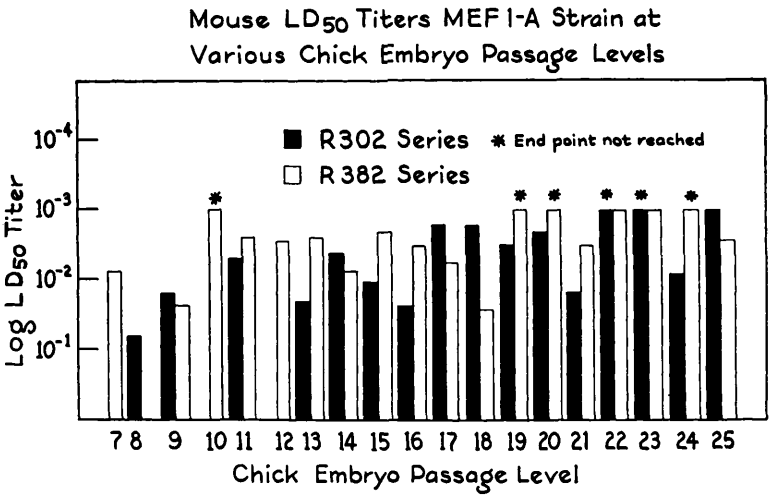

FIG. 1.

been injected intracerebrally either with chick embryo suspensions, or with cord tissue taken from monkeys that had been injected intracerebrally with chick embryo suspensions. The results of the tests done at the 7th, 9th, 10th, 12th and 21st chick embryo passage levels of the R-302 series are summarized in Table I. The data show that the viral agent under test was appreciably neutralized by the Lansing type immune monkey serum prepared in our laboratory, as well as by those sera received from Dr. Joseph Melnick and Dr. Howard Howe of Yale and Johns Hopkins Universities, respectively. These findings strongly indicate the identity of the R-302 series virus as Lansing type human poliomyelitis virus.

The identification of the virus was further supported by the results obtained in virulence tests in Rhesus monkeys. On May 9, 1952, 2 Rhesus monkeys (2135 and 2136) were inoculated with a 66% chick embryo suspension of the 7th consecutive egg passage of the R-302 series. Monkey 2135 was injected intracerebrally with 0.5 ml of this suspension, while monkey 2136 was inoculated intramuscularly with 1.0 ml of the same material. Seven days later, the monkey injected intracerebrally (2135) showed signs of weakness of the right leg, accompanied by noticeable general tremor. The next day the animal showed complete paralysis of both legs and right arm. Later that day, it was sacrificed and its spinal cord removed. Part of the spinal cord was fixed in 80% alcohol for histopathologic examination† and the remainder was made into a 10% tissue suspension, which was inoculated intracerebrally into 8 Swiss mice; 7 of these became paralyzed between the 8th and 15th day after inoculation.

A suspension of the brain and spinal cord tissues from 2 of these paralyzed mice was tested by neutralization procedure against normal monkey serum and the Lederle anti-Lansing monkey serum. The results, shown in Table I, strongly suggested that the passage virus was a Lansing type strain of human poliomyelitis virus.

Monkey 2136 remained symptom-free following intramuscular injection and serum obtained 68 days after inoculation failed to show neutralizing antibodies.

A tissue suspension having a mouse LD_{50} titer of $10^{-2.3}$, prepared from the 11th chick embryo passage of the R-302 series was titrated intracerebrally in monkeys. The undiluted tissue suspension and 10^{-1} and 10^{-2} dilutions thereof were injected intracerebrally in duplicate monkeys (Table II). Only one of the 6 monkeys (2245) developed symptoms of poliomyelitis and it recovered.

† Dr. George A. Jervis, who performed the histopathologic study, reported typical cord lesions of poliomyelitis.

TABLE I. Chick Embryo Adapted MEF1 Strain of Poliomyelitis Virus. Results of neutralization tests.

Passage series	Virus passage level	Mouse LD_{50} titers and neutralization indices	Serum used: Normal monkey	Immune (Lederle)	Immune (Y-SK)*	Immune (Armstrong)†	Immune (GDVII)
	7 E + 1 monkey (2135) + 1 mouse	LD_{50}	$10^{-3.5}$	$10^{-2.2}$			
		N.I.		47			
R-302	9 E + 1 mouse	LD_{50}	$10^{-2.66}$	$10^{-1.5}$			
		N.I.		20			
	10 E + 1 mouse	LD_{50}	$10^{-3.2}$	$<10^{-1.3}$			
		N.I.		>97			
	12 E + 1 mouse	LD_{50}	$10^{-3.3}$	$10^{-1.7}$	$10^{-1.3}$		
		N.I.		74	100		
	21 E	LD_{50}	$10^{-1.7}$			10^{-0}	
		N.I.				>84	
	7 E + 1 mouse	LD_{50}	$>10^{-3.0}$	$10^{-1.0}$			
		N.I.		>100			
	9 E + 1 mouse	LD_{50}	$>10^{-3.8}$	$10^{-2.0}$			$>10^{-3.8}$
		N.I.		63			0
R-382	11 E + 1 monkey (2312) + 1 mouse	LD_{50}	$10^{-3.4}$	$10^{-1.6}$			
		N.I.		63			
	17 E	LD_{50}	$10^{-2.0}$			10^{-0}	
		N.I.				>100	

7 E + 1 monkey + 1 mouse = 7 embryo passages + 1 monkey brain passage + 1 mouse brain passage used as mouse brain suspension.
9 E + 1 mouse = 9 embryo passages + 1 mouse brain passage used as mouse brain suspension.
21 E = 21st embryo passage used as embryo suspension.
* Lansing immune monkey serum received from Dr. Joseph L. Melnick of Yale University.
† " " " " " " Dr. Howard A. Howe of Johns Hopkins University.

The sera of monkeys 2245 and 2246, both of which had received the undiluted inoculum, as well as the serum of monkey 2250, which had received the 10^{-2} dilution intracerebrally, were shown to contain neutralizing antibodies by a qualitative test carried out with a virus suspension derived from the 64th hamster passage of Moyer's MEF1-A strain. In a subsequent quantitative neutralization test carried out with Moyer's 131st hamster passage of the MEF1-A strain of virus, neutralization indices of 31,620 and 39,810 were obtained for the sera of monkeys 2245 and 2246, respectively. The serum of monkey 2250 was not tested quantitatively. All 3 monkeys (2245, 2246 and 2250) were challenged intracerebrally with Lansing monkey cord pool virus 68 days after original inoculation, and all 3 survived without showing any symptoms of poliomyelitis. In contrast, all of the 13 normal, control monkeys used in the test came down with typical poliomyelitis 7 to 15 days after intracerebral injection.

The 11th chick embryo passage virus of the R-302 series was shown thereby to induce in monkeys the formation of antibodies which neutralized the MEF1-A strain virus in significant degree and produced in such animals a resistance capable of withstanding direct intracerebral challenge with at least 700 monkey MLD's of virulent virus of the Lansing monkey cord pool.

The remaining 3 monkeys used in the titration (2247, 2248 and 2249) apparently failed to develop either symptoms or antibodies and were not challenged intracerebrally.

Chick embryo series R-382. This series was initiated in 7-day-old chick embryos inoculated by way of the yolk sac on February 13, 1952, with a 10% brain and cord suspension prepared from the 3rd mouse passage of Moyer's 119th hamster passage of the MEF1-

TABLE II. Identification of the Chick Embryo Propagated Virus by the Response of Monkeys to Inoculation.

Series No.	Chick embryo passage	Monkey No.	Dilution used	LD_{50} titer in mice	Vol., ml	Route	Date	Symptoms	Monkey challenge		Mouse neutralization tests	
									I.C.	Date	Qualitative M/R‡	Quantitative N.I.§
R-302	7	2135	Undil.	N.D.	.5	I.C.	5/9/52	Yes*	—		—	—
		2136	"	"	1	I.M.	"	No	N.D.		7/8	—
		2245	"	$10^{-2.80}$	.5	I.C.	6/5/52	Yes†	P	8/12/52	0/8	31620
		2246	"	"	"	"	"	No	P		0/8	39810
	11	2247	10^{-1}	"	"	"	"	"	N.D.		8/8	—
		2248	"	"	"	"	"	"	N.D.		8/8	—
		2249	10^{-2}	"	"	"	"	"	N.D.		8/8	—
		2250	"	"	"	"	"	"	P		0/7	—
R-382	9	2253	10^{-1}	$10^{-1.04}$	"	"	"	"	P	8/12/52	0/8	21380
		2254	"	"	"	"	"	"	P		7/8	631
	11	2312	Undil.	N.D.	"	"	6/30/52	Yes*	—	8/12/52	—	—
		2313	"	$10^{-2.87}$	"	"	"	No	P		3/8	25120

13 normal, control monkeys inj. intracer. with Lansing monkey cord pool virus on 8/12/52; all developed typical poliomyelitis within 7 to 15 days.‖

I.C. = Intracerebral; I.M. = Intramuscular; N.D = Not done; P = Protected.
* Weakness followed by paralysis of the extremities 7 days after inoculation. Lansing type virus recovered from the cord. Histologically typical poliomyelitis.
† General tremors and right arm weakness 7 days after inoculation. Monkey recovered after 2 weeks.
‡ Qualitative neutralization tests performed with the 64th hamster passage of the MEF1-A strain of virus, expressed as the mortality ratio = M/R.
§ Quantitative neutralization tests performed with the 131st hamster passage of the MEF1-A strain of virus, expressed as the neutralization index = N.I.
‖ Challenge dose represented 700 MLD_{50}'s of the Lansing pooled monkey cord tissue.

A strain. The live embryos were harvested 7 days later, and a 66% tissue suspension prepared from them was inoculated intracerebrally in a group of 4 mice designated R-382. From the paralyzed mice of R-382 a 10% suspension of pooled brain and spinal cord tissue was prepared and injected into the yolk sacs of 13, 7-day-old chick embryos on March 17, 1952. Since that date the R-382 series has been maintained continuously in chick embryos by yolk sac inoculation for 38 generations and the passage series is still in progress. Groups of 3 to 7 mice were injected intracerebrally with tissue suspensions at each passage level as a qualitative test for the presence of virus. The inoculated mice invariably developed paralysis and died. Tissue suspensions from the 7th, 9th and all subsequent chick embryo passages were titrated intracerebrally in mice. The results of these titrations are presented graphically in the accompanying figure.

Neutralization tests for identification of the passage virus were done with tissue suspensions representing the 7th, 9th, 11th and 17th chick embryo passages. The results, summarized in Table I, show that the virus at the first 3 passage levels of this sequence was neutralized by Lederle anti-Lansing monkey serum and that the virus at the 17th chick embryo passage was neutralized by the anti-Lansing monkey serum received from Dr. Howe. In addition, the 11th embryo passage virus was not neutralized by the immune serum prepared against the GDVII strain of mouse encephalomyelitis virus. These neutralization tests indicate that the R-382 series chick embryo passage virus is a Lansing type strain of human poliomyelitis virus.

This identification is substantiated by the observations made on monkeys 2312 and 2313 (Table II), which were injected intracerebrally with a 66% chick embryo tissue suspension having a mouse LD_{50} titer of $10^{-2.6}$ prepared from the 11th chick embryo passage. Monkey 2312 showed typical paralytic symptoms on the 7th day after inoculation. It was sacrificed the following day and sections of its spinal cord revealed typical poliomyelitic lesions.† Virus recovered from the cord of this animal was neutralized by Lederle anti-

Lansing monkey immune serum (Table I). Monkey 2313 survived without the development of symptoms, but a postinoculation serum sample showed a neutralization index of 25,120 when tested against Moyer's 131st hamster passage of MEF1-A strain virus.

Similarly, monkeys 2253 and 2254, which were injected intracerebrally with 0.5 ml each of a 6% chick embryo suspension of the R-382 series 9th passage, failed to show any signs of illness but developed neutralization indices of 21,380 and 631, respectively, when tested against Moyer's 131st hamster passage MEF1-A virus. These 3 monkeys (2253, 2254 and 2313) were later challenged intracerebrally (Table II) with Lansing monkey cord pool virus and all 3 survived without showing any symptoms of poliomyelitis, whereas all of 13 normal, control monkeys used in the test developed typical poliomyelitis within 7 to 15 days.

The chick embryo passage virus of the R-382 series was thus shown to parallel that of the R-302 series at the same passage levels. It produced typical symptoms and cord lesions of poliomyelitis in inoculated monkeys; it stimulated the formation of antibodies against the MEF1-A strain virus and induced resistance in monkeys to intracerebral challenge with virulent Lansing type monkey cord pool virus.

Observations on the passage virus in chick embryos. After approximately 40 serial passages in the chick embryo the virus neither kills infected embryos nor causes superficial gross lesions by which its presence and multiplication can be judged.

Using the yolk sac route of inoculation and titrating tissues and fluids 6 to 8 days later, the highest concentrations of virus have been found in the body of the embryo. Testing of tissue suspensions prepared from decapitated embryos gave virus titers comparable to those obtained with complete embryos, which suggests that virus multiplication is not restricted to the nervous tissues. Only traces of virus have been demonstrated in yolk sac or allantoic membrane suspensions.

Attempts to adapt the MEF1-A strain of virus to the chick embryo at the 70th, 80th, 100th and 109th hamster passage levels have not been successful to date. The 115th hamster passage virus, however, has now been established in chick embryos. Thus the experience in adapting the MEF1 strain of poliomyelitis virus to the chick embryo closely parallels that of Schlesinger(24) in adapting dengue virus to growth in the chick embryo.

These observations suggest that between the 109th and the 115th hamster passage a virus mutant appeared which was able to grow in the chick embryo. Such an interpretation is also consistent with the apparent failure of other investigators to adapt other strains of human poliomyelitis virus to growth in this medium.

Summary. 1. The MEF1-A strain of human poliomyelitis virus(10), after 119 serial passages in suckling hamsters, has been maintained in one passage series for 41 consecutive generations and in a second series for 38 generations in the developing chick embryo by the yolk sac route of inoculation. Both lines of passages are still in progress. 2. The identity of the chick embryo adapted virus with human poliomyelitis virus was established by mouse neutralization tests in which anti-Lansing type immune sera from 3 different sources were used; by the ability of the passage virus to stimulate the formation of antibodies in monkeys which neutralize known virulent strains of Lansing type poliomyelitis virus; and by the ability of monkeys to withstand intracerebral challenge with known virulent strains of poliomyelitis virus after they had survived inoculation with the chick embryo passage virus. The identity of the passage virus was further established by its ability to induce typical symptoms and histopathologic lesions in monkeys following intracerebral inoculation.

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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)

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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.