Adaptation of Leon Strain of Poliomyelitis to Mice. (19032)

C. P. Li* and K. Habel.

From the National Microbiological Institute, National Institutes of Health, U. S. Public Health Service, Federal Security Agency.

The increased amount of experimental work in the field of poliomyelitis made possible by Armstrong's successful adaptation of the Lansing strain to rodents is well known(1). Since that time a number of attempts have been made by workers in the poliomyelitis field to repeat this adaptation with other strains. Thus far the only successful results have apparently been obtained with strains which are identical with or immunologically related to the Lansing strain(2-5). Milzer and Byrd(6) reported the successful adaptation of Leon virus to mice using autolyzed brain as an adjuvant in the intracerebral inoculation of mice. This Leon mouse virus is not currently available and further attempts by these workers to repeat this adaptation have thus far been unsuccessful. More recently, however, they have found that "*Salmonella typhimurium* is apparently responsible for the previously reported enhancing effect of autolyzed brain tissue diluent on Lansing virus. Experiments using *S. typhimurium* vaccine for adaptation of strains of poliomyelitis virus to mice are now in progress."†

A technic was recently developed in this laboratory whereby Lansing virus was inoculated directly into the spinal cord of mice(7). It was shown that mice were more susceptible to Lansing infection by this route than by the usual intracerebral method of inoculation. It seemed logical to once more attempt to adapt non-Lansing strains to mice by intraspinal inoculation. This report presents evidence that the Leon strain of poliomyelitis has been thus adapted to mice.

Materials and methods. Leon virus. The Leon virus was obtained from the American Type Culture Collection and had originated in the laboratory of Dr. H. A. Howe of Johns Hopkins Poliomyelitis Center. It was received in its 23rd monkey passage and was given one passage intracerebrally, at a 10^{-3} dilution in a *M. mulatta* monkey in our laboratory before being used in mice. *Immune monkey sera.* Samples of proven Leon, Lansing, and Brunhilde immune monkey sera were obtained through the courtesy of Dr. H. A. Howe from the Johns Hopkins Poliomyelitis Center, Dr. J. Melnick at Yale University, and Dr. W. McD. Hammond at the University of Pittsburgh. In our own laboratory, monkeys were hyperimmunized with 2 cc doses of 20% whole emulsion of monkey cord virus given intramuscularly twice weekly for approximately 14 doses and bled 10 days after the last dose. Some of these sera were not checked for antibody content in monkeys before being used in mouse tests. *Intraspinal inoculation.* The technic of intraspinal inoculation has been previously described.

Virus neutralization test. All sera were inactivated at 56°C for 30 minutes before being serially diluted and mixed with equal parts of the supernatant fluid from a 20% emulsion of Leon mouse passage spinal cords. The mixtures were placed at 4°C for 4 hours before being inoculated intraspinally into mice. Normal monkey serum was included as a control and virus titers were carried out after the 4 hours incubation in the presence of 1/4 dilution of normal serum. In some tests in-

* National Institutes of Health Research Fellow.

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cubation for 30 minutes at room temperature preceded the ice box incubation.

Animals. Swiss and C_3H mice were used at the age of 3-4 weeks. *Macacus mulatta* monkeys weighing 5-7 lb were used for infectivity tests. Cotton rats were usually young adults and weighed approximately 70 g at the time of inoculation.

Results Primary isolation—8 C_3H mice were inoculated intraspinally with 0.02 ml of a 20% whole emulsion of spinal cord from Leon monkey I-21. There was one death without preceding paralysis on the 7th day. On the 15th day, 2 mice were paralyzed in the hind legs. On the 16th day one mouse was found dead. On the 18th day one mouse was paralyzed in the right front leg, one in the hind leg on the 19th day, 2 others were found dead on the 20th day and the last mouse was found dead on the 21st day. All mice observed with paralysis were sacrificed and their cords harvested for passage.

Intracerebral inoculation of C_3H mice with the same original monkey cord suspension did not cause any paralysis although there were some deaths between the 5th and 11th days. On the 14th day 3 mice developed non-specific symptoms of weakness and ruffled fur and their brains were harvested. Subsequent intracerebral passage of these brains in C_3H mice gave negative results.

Mouse passages. The cords of all mice showing definite paralysis in the first passage were pooled and the supernatant fluid of a 10% emulsion passed in C_3H mice intraspinally. To date this virus has been through a total of 35 such intraspinal mouse passages. The first 5 passages were made in C_3H mice but all subsequent work has been done in Swiss mice which appear to be equally susceptible to the passage virus. Table I shows the morbidity rates and average incubation periods for the early passages.

Identification of passage virus as Leon poliomyelitis. Besides serological and cross immunity experiments to establish the identity of a strain of poliomyelitis other criteria are widely accepted as proof that a particular poliomyelitis virus is probably of human origin. These criteria include its infectivity for monkeys, the typical histopathology produced in the monkey spinal cord and finally its ability to be neutralized by pooled adult human serum. The mouse passage Leon virus has been checked by each of these methods.

TABLE I. Incubation Period and Morbidity of Mouse Passages of Leon Virus. Passage of 10-1 dilution of mouse cords.

Mouse passage	Incubation period	Morbidity
1	15	100
2	4	70
3	4	100
4	4	100
5	5	90
6	5	85
7	4	100
8	5	100

(a) *Infectivity for monkeys.* Four monkeys received 1 ml intracerebrally and 1 ml intranasally under ether anesthesia of a 20% whole emulsion of mouse passage No. 8 cords. Fig. 1 shows the subsequent illness in these monkeys. Two out of the 4 developed fever, tremors and finally paralysis of at least 2 extremities after 6 and 7 days' incubation period. These 2 animals when prostrated were sacrificed for virus and histological examination. The other 2 monkeys developed fever and tremors between the 7th and 9th days after inoculation and one (K-19) had weakness of the right arm which did not progress. Cord from monkey K-18 was inoculated intracerebrally at a 10% emulsion into 2 monkeys. After 5 and 9 days these animals came down with fever, tremors and prostrating paralysis. Cords from K-18 and K-21 representing the first monkey passage of mouse virus were examined by Dr. James Peers of the Laboratory of Pathology and found to have typical histopathological lesions of polio of moderate severity at thoracic, lumbar and sacral levels. Subsequent tests for infectivity after intracerebral inoculation of monkeys have been carried out with pooled mouse passages 20, 21, and 22 in an attempt to quantitate and compare the titer intracerebrally in monkeys to intraspinally in mice. Table II shows that if any difference existed in the susceptibility of mice and monkeys to mouse passage Leon virus perhaps monkeys

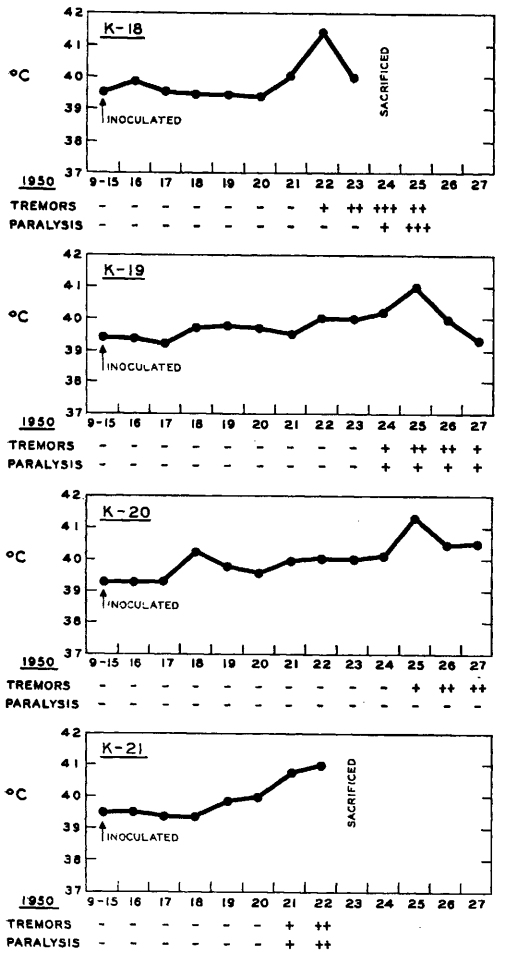

FIG. 1. Results of intracerebral inoculation of monkeys with 8th mouse passage Leon.

were slightly more susceptible. Cord of monkey K-18 (1st monkey passage of mouse virus) was inoculated intraspinally into C_3H mice and the virus thus reisolated in mice and carried through 9 subsequent mouse passages.

(b) *Cross immunity tests in monkeys.* Cord emulsion from first monkey passage of mouse Leon virus was inoculated into 2 fresh monkeys (K-18-2A and K-18-2B) and monkey I-50. Monkey I-50 had previously been hyperimmunized with monkey passage Leon virus and shown to be immune to an intracerebral challenge with the homologous monkey virus. Fig. 2 shows that both fresh monkeys came down with severe paralytic disease while the Leon immune monkey remained normal. A cross-check of the immunity produced by mouse passage Leon virus in monkeys when challenged by monkey passage Leon virus was next carried out. Monkeys K-19 and K-20 which had survived inoculation with mouse passage No. 8 virus with minimum symptoms (see above) were challenged with Leon monkey passage virus together with 2 fresh monkeys (K-39 and K-40). A 10^{-3} dilution of monkey passage No. 24 (monkey I-22) cord was inoculated intracerebrally. Fig. 3 shows that the 2 fresh monkeys developed typical paralytic disease after 6- and 9-day incubation periods, whereas the 2 monkeys convalescent from infection with mouse passage Leon virus failed to develop any symptoms whatsoever. These 2 monkeys had been bled prior to this challenge and both had demonstrable neutralizing antibodies against the mouse passage Leon virus in their serum.

(c) *Virus neutralization tests with mouse passage Leon virus.* The mouse passage Leon virus was tested against serum from a monkey that had been hyperimmunized with monkey passage Leon cord suspensions and proven immune to intracerebral challenge with this same virus. Results are given in Table III. This type of neutralization test was repeated several times with later passages of mouse virus against Leon immune monkey serum obtained from other laboratories with approximately the same quantitative results. Tests against anti-Theiler's virus serum and anti-EMC serum (kindly supplied by Dr. Joel Warren of the Army Medical School) run at the same time were completely negative. Likewise tests against Lansing and Brunhilde immune monkey serums have been negative except in several tests there appeared to be some slight neutralization of mouse passage Leon virus by Lansing immune serum but quantitatively not as great as with Leon serum. Several tests with commercial samples

TABLE II. Titration of Mouse Passages 20, 21 and 22 Leon Virus IS in Mice and IC in Monkeys.

		Dilution of inoculum		
		10^{-1}	10^{-2}	10^{-3}
Mice	IS	4/8*	2/17	0/8
Monkeys	IC	2/2	1/2	1/2

* No. of animals paralyzed over No. inoculated.

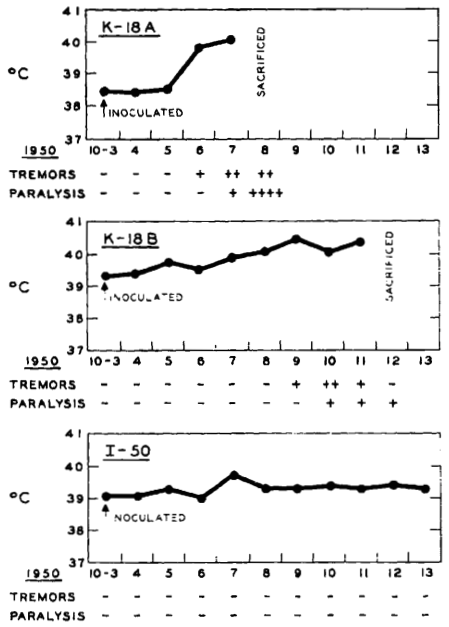

FIG. 2. Intracerebral challenge of 2 fresh monkeys (K 18 A and K 18 B) and one Leon immune monkey (I 50) with 1st monkey passage of Leon mouse adapted virus (monkey K 18 cord).

of gamma globulin (human) which had been shown to contain good neutralization indices against Lansing virus were tested for neutralization of mouse Leon virus but in most instances gave equivocal results. The best index that was obtained was between a 1/4 and a 1/16 dilution of the gamma globulin against 1 to 5 minimum paralyzing doses of mouse Leon virus. A typical protocol for these various neutralization tests is shown in Table IV.

It was felt that these neutralization tests carried out by intra-spinal inoculation of mice were not as clear cut as desired so a neutralization test intracerebrally in monkeys was done using Leon immune monkey serum (obtained from W. McD. Hammond) and normal monkey serum mixed with mouse passage Leon virus (M.P. No. 27) and with monkey passage Leon virus. Undiluted inactivated serum was mixed with supernatant of 20% mouse cord emulsion and 10% monkey cord emulsion. For the monkey virus this represented approximately 100 MPD for monkeys. After 30 minutes at room temperature and 3 hours at 4°C, 1 cc amounts were inoculated intracerebrally into 4 monkeys and in the case of the mouse virus also into groups of mice. Table V shows the results in monkeys and the protocol in Table IV includes the results in mice. The monkey test gave good neutralization of the mouse virus with serum prepared in another laboratory by hyperimmunization with monkey Leon virus.

(d) *Chick embryo inoculation.* Mouse passage No. 10 at a 10% emulsion was inoculated into the yolk sac of 6-day and allantoic sac of 8-day chick embryos. Every 7 days the yolk sac series was passed and transferred while the allantoic sac group were passaged at 6-day intervals. At each passage whole embryos, portions of chorio-allantoic and amniotic membranes and yolk sacs were emulsified in allantoic fluid for the next inocu-

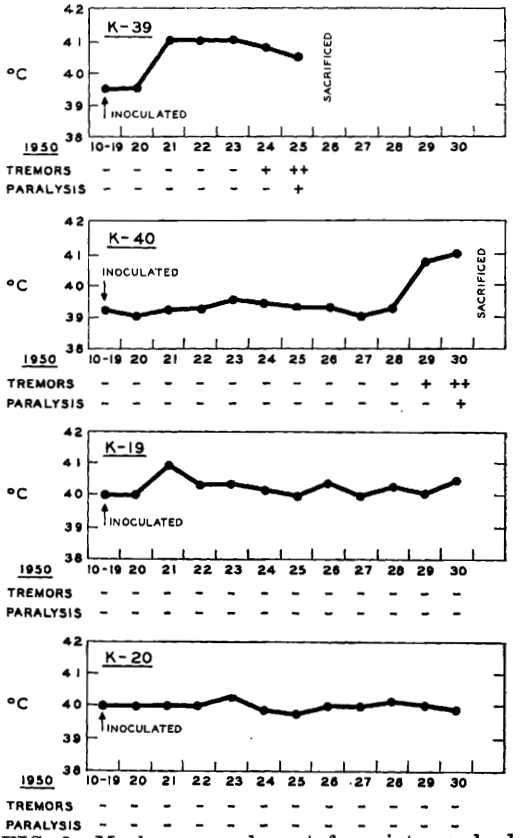

FIG. 3. Monkeys convalescent from intracerebral inoculation with mouse passage virus (K 19 and K 20) and two fresh monkeys (K 39 and K 40) challenged IC with 10^{-3} dilution of monkey passage Leon virus (I 22 cord).

TABLE III. Neutralization Test with Mouse Passage Leon Virus Against Serum of Monkey Immunized with Monkey Passage Leon Virus (IS in Mice).

	Serum dilutions*		
	Undil.	1/4	1/16
Normal monkey	6/9†	5/9	3/8
Leon immune monkey	0/7	1/7	3/10

* Dilutions of serum mixed with 1/5 dilution of mouse passage 9 cords and held at 37°C for 1 hr.
† No. of mice paralyzed over No. inoculated.

TABLE IV. Neutralization Test of Mouse Passage Leon Virus Against Monkey Immune Serum and Human Gamma Globulin (IS in Mice).

	Serum dilutions*			
	Undil.	1/4	1/16	1/64
Normal monkey	7/8†	9/10	7/8	—
Brunhilde immune monkey	7/8	6/6	7/9	—
Lansing " "	7/10	3/5	7/9	—
Leon " "	3/10	3/7	7/9	—
Gamma globulin	—	2/8	5/9	6/8
Virus titer 1/50‡	—	5/9	—	—
1/250	—	4/10	—	—

* Dilutions of serum mixed with 1/5 dilution of M.P. 27 Leon virus and incubated 30 min at room temperature then 3 hr at 4°C before IS inoculation of mice.
† No. of paralyzed over No. of inoculated mice.
‡ After incubation in 1/4 normal serum serial dilutions made in same diluent for virus titration.

TABLE V. Neutralization Test with Leon Immune Monkey Serum Against Mouse and Monkey Passage Leon Viruses IC in Monkeys.

Virus	Monkey serum*	
	Normal	Leon immune
Mouse passage virus†	3/4	0/4
Monkey " " ‡	4/4	1/4

* Undiluted inactivated serum mixed with virus and incubated 30 min at room temperature then 3 hr at 4°C before IC inoculation of 1 cc of mixture in monkeys.
† 1/5 dilution mouse passage #27.
‡ 1/10 " monkey K-39 cord.

lation. In 3 such blind passages there were no specific deaths and no pathological lesions were produced in the chick embryos or membranes. The third passage materials were injected intraspinally into groups of mice with negative results.

Properties of Leon mouse passage virus. (a) *Incubation period, mortality, type of paralysis.* The incubation period in the first mouse passage was 15 days but from the second passage on has been between 4 and 6 days at a 10^{-1} inoculum. After the twentieth passage an occasional mouse would develop paralysis on the third day. After paralysis develops the subsequent mortality varies quite a bit from one passage to another but it has not been unusual for 20% of the paralyzed mice to survive. The total morbidity has varied also from 50% to 100%. In more recent passages the morbidity and mortality have tended to be higher and more consistent. In neutralization tests there is a tendency for a greater proportion of the mice inoculated with serum-virus mixtures to survive after developing paralysis than in routine passage mice. The relative proportion of front leg to hind leg paralysis after intraspinal inoculation with this Leon virus is greater than in mice inoculated by this route with Lansing virus.

(b) *Titration of mouse virus.* Virus titers of mouse cords by the intraspinal inoculation in mice have been consistently low, usually a 50% mortality not being obtained beyond a 1/50 dilution of cords. All attempts to infect mice intracerebrally have thus far been negative, yet this same virus emulsion promptly and readily caused typical poliomyelitis after intracerebral inoculation of monkeys. Likewise attempts to infect cotton rats by intracerebral inoculation have been negative. Intraspinal inoculation of cotton rats has not given consistent results although paralytic disease has been produced several times. In each instance inoculation of paralyzed cotton rat cord emulsion intraspinally into mice was negative. Attempts at cotton rat passage have been equivocal. One attempt at intraspinal infection of cotton rats with monkey Leon virus gave mild paralytic symptoms which could not be maintained on passage.

Repeat of primary adaptation to mice. Cord of monkey 122 representing the same monkey passage of Leon virus as used in the original adaptation experiment but from a different monkey was used to inoculate mice intraspinally. The first group of C_3H mice had 2 paralyzed out of 10 on the 30th day. Second passage in C_3H mice had 5 paralyzed out of 12 with the earliest symptoms on the 9th day. Starting with the 3rd passage, Swiss mice were used and the incubation shortened to 5 days. This substrain has been passed through

16 passages intraspinally in mice and has continued to have an incubation period of 5 to 6 days. Mouse passage 15 cords were inoculated intracerebrally into 2 monkeys (K80 and K81) in a 20% emulsion. Both monkeys developed fever, marked tremors and hind leg paralysis starting on the 7th day.

Discussion and summary. Leon type of human poliomyelitis virus has been adapted to mice from original monkey cord virus by use of an intraspinal technic of inoculation. This mouse adapted Leon virus has been serially passed through mice for 35 passages and causes flaccid paralysis, more frequently in the front legs, after an incubation period of 4 to 6 days. A second similar adaptation has been accomplished and this strain carried through 16 mouse passages. The mouse virus causes paralysis only after intraspinal inoculation of mice, being negative when given intracerebrally and is of low virulence thus far. Apparently this virus is still as virulent for monkeys as it is for mice. Proof that this mouse virus is indeed Leon poliomyelitis virus rests on its ability to cause typical clinical and histopathological disease after intracerebral inoculation of monkeys, cross immunization against monkey Leon virus in monkeys and cross neutralization tests in mice and in monkeys. The virus also fails to propagate in the chick embryo. It is neutralized by human gamma globulin in low dilutions. Thus far attempts to adapt the Brunhilde type of human poliomyelitis virus to mice by this same method have been completely negative.

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Megaloblastic Anemia in Pregnancy. Remission Following Combined Therapy with Ascorbic Acid and Vitamin B_{12}.* (19033)

ROY G. HOLLY. (Introduced by C. D. May.)

From the Department of Obstetrics and Gynecology, University of Minnesota Medical School, Minneapolis.

A recent report by Thompson and Ungley (1) has reviewed many of the clinical and hematologic features of megaloblastic anemia in pregnancy. These authors emphasize the lack of response to vit. B_{12}. Similar observations had previously been reported by Day, Hall and Pease(2). In this respect the megaloblastic anemia in pregnancy is similar to megaloblastic anemia experimentally produced in monkeys(3) and to the megaloblastic anemia in some infants(4). Remissions are regularly produced in these three as well as other megaloblastic anemias by folic acid therapy. The megaloblastic anemia in some infants and as experimentally produced in monkeys is related to a deficiency in ascorbic acid and can be prevented by a normal intake of this substance. May(3) has suggested that the ascorbic acid deficiency may interfere with the conversion of folic acid to the citrovorum factor as Nichol and Welch(5) have shown that ascorbic acid augments the conversion of folic acid to the citrovorum factor *in vitro*. It is entirely possible that megaloblastic anemia in some infants and that produced in monkeys results from a deficiency of the citrovorum factor which in turn is produced by altered folic acid metabolism resulting from ascorbic acid deficiency.

Observations on 5 patients with megalo-

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