The Institute for Infectious Diseases, The University of Tokyo

Experimental Study on Measles Virus

4th Report: Adaptation of the Virus to Mouse-brain, especially after Serial Passages through Chick Embryo Cultures

By

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(*Received June 5, 1959*)

Introduction

Since *Arakawa* [1948 (1), 1949 (2), 1954 (3)] succeeded in isolation of the measles virus by the serial intracerebral inoculations into young mice, *Arakawa* et al. (4) (1955), *Wada* and *Ogawa* (5) (1955), *Fujita* (6) (1955), *Suzuki* et al. (7) (1958), *Imagawa* and *Adams* (1958) reported successful results with the same method.

Also the successful chick embryo culture of the virus is reported by *Torres* and *Teixeria* (9) (1955), *Taniguchi* et al. (10) (1935), *Wenckebach* and *Kunert* (11) (1937), *Degkwitz* and *Mayer* (12) (1937), *Heinzman* (13) (1939), *Rake* and *Shaffer* (14) (1939), *Nishigame* (15) (1942), *Arakawa* (2) (1949), *Enders* and *Peebles* (16) (1954). Among the rest *Arakawa* facilitated the adaptation of the virus to mouse-brain by the preparatory passages on to developing chick embryo. The present paper is concerned with the further outcomes obtained by this method.

Material and Methods

Virus: Patient's serum was taken within 24 hours after an attack, except the sera of Yamasaki and Sakurai, which were taken 24 to 48 hours after. Of the materials used in 1955, one was the pool of three patients' sera. The others were each from a single patient.

Fertile hen's eggs incubated at 37° C for 9—10 days were inoculated on to the chorio-allantoic membranes with 0.2 ml. of the virus material and then incubated at 36° C for 4—5 days. In order to observe the changes on the chorio-allantoic membrane, *Tanaka's* method, which makes an opening at the obtuse

end of the egg standing upright was employed. The chorio-allantoic membranes were removed aseptically and ground in a mortar with sand or hard glass powder, then made a 20 percent emulsion with saline solution (pH 7.0) and centrifuged at 3,000 rpm. for 20 minutes. The supernatant fluid thus obtained was used for serial passages. But in case of Watanabe strain, the 16th passage was ultracentrifuged at 40,000 rpm. (about 100,000 g) by Spinco L rotor # 40 for 60 minutes, the sediment of which was resuspended in saline of 1/20th of the original volume, and centrifuged at 3,000 rpm. for 20 minutes, the supernatant was used for mouse-inoculation or further passage on to the chick embryo.

Mice used for isolation or passage of the virus weighed 6 to 7 g and to each one of them 0.025 ml. of the material was inoculated intracerebrally. In case of Hasegawa-strain and its control, 10 litter mice were divided into two groups and used. Symptoms were then carefully observed, and on confirming the severe symptoms or death, the brains were removed and ground in a mortar with sand or hard glass powder, added with saline and centrifuged at 3,000 rpm. for 15 minutes in the same manner as in the case of infected chorio-allantoic membrane.

Sterility test: thioglycollate medium or blood agar medium and liver-liver broth were used, incubating at 37° C for two weeks.

Neutralization test: 0.5 ml. of the serial tenfold dilutions of supernatant fluid of the 10 percent emulsion of infected mouse-brain was mixed with 0.5 ml. of the serum inactivated by heating at 56° C for 30 minutes, and incubated at 37° C for 2 hours, then kept overnight in an ice box. Normal rabbit serum was used for the control groups. A dose of 0.025 ml. was used for inoculation. Four mice each weighing about 10 g were used for each series. LD_{50} was calculated by the formula of *Kärber*.

Complement fixation test was carried out in accordance with the method of *Kolmer*. A 10 percent saline emulsion of infected mouse-brain was slightly centrifuged, and the supernatant fluid was frozen and thawed five times and centrifuged at 5,000 rpm. for 30 minutes, and the supernatant fluid made the antigen. Serum was inactivated by heating at 56° C for 30 minutes before use. The mixture of antigen and serum in 0.25 ml. each, added with two full units of guinea pig complement in 0.5 ml., were incubated at 37° C for 30 minutes and after keeping overnight at 4° C, 0.5 ml. of the hemolytic system was added, this was the case in 1957. However, in the case of 1955, incubation at 37° C has been omitted. The hemolytic system consisted of 0.25 ml. of a 3 percent sheep cell suspension and 0.25 ml. of sheep cell hemolysin containing 3 units. The test was read after final incubation at 37° C for 30 minutes. The point which by inspection shows approximately half the cells hemolyzed (2+) made the titration end-point. Control antigen was prepared from the normal mouse-brain by the same technic as mentioned above.

To obtain the *antiserum for neutralization test*, rabbits weighing 2,200 g were inoculated subcutaneously with 1, 2, 4, 8, 10, 32 and 60 ml. respectively of the supernatant fluid of the 10 percent emulsion of infected mouse brain at an interval of a week. The above was the case in 1955. But in 1957, a 8-month-old pony was inoculated with the following 3 kinds of suspension of infected hen's chorio-allantoic membrane and embryo: A 10 percent emulsion of chorio-allantoic membrane and embryo, infected with Ohki-strain was centrifuged at 3,000 rpm. for 20 minutes to obtain the supernatant fluid. This fluid (1st antigen) was centrifuged by Sharpless centrifuge for 60 minutes. The

sediment was suspended in the saline of 1/25th (2nd antigen) and 1/100th (3rd antigen) of the original volume respectively. The total amount of the fluids inoculated was as follows: 2,600 ml. of 1st antigen and 1,590 ml. of 2nd antigen subcutaneously; 100 ml. of 2nd antigen intracutaneously; 315 ml. of 1st antigen and 109 ml. of 3rd antigen intravenously. To obtain the *standard serum for complement fixation test*, guinea pigs weighing 500 g were inoculated intracerebrally with 0.1 ml. of the supernatant fluid of 10 percent saline emulsion of infected hen's chorio-allantoic membrane twice at an interval of a week. The antiserum was taken at a week after final immunization in each case.

Results

1. *Isolation of virus by mouse-intracerebral inoculation after its propagation in fertile hen's embryo.*

The results of virus isolation are shown in Table 1.

As shown in the table, in the case of Watanabe-strain, 10 percent emulsion of infected chorio-allantoic membrane from 16th generation was concentrated 20 times, passaged further in fertile eggs and 10 percent emulsion of infected chorio-allantoic membrane from 17th generation was inoculated intracerebrally into mouse, then the virus was fixed to mouse at once. But in case of "Enomoto" and "Pooled" strains, such a rapid fixation was not attained. From eight patients' materials, 5 strains (Yokoo, Iwao, Hasegawa, Kobayashi and Midori) were isolated after

Table 1. Adaptation of virus to mouse brain after serial passages in fertilized eggs

Year	Strain	Egg-material			Mice (intracerebral inoculation)
		Number of eggs*	Number of passages	Harvesting time (days)	
1955	Watanabe	1/3	3	5	△ △ ○ ○ 2 2
		2/3	8	5	● ○ ○ ○ 7
		3/3	14	5	⊘ ⊘ ⊘ ⊘
		3/3	16	5	● ● ○ ○ ○ †6 7
		2/3 †	17	5	● ● ● ● ● 5 5 5 5 5
	Enomoto	1/3	8	5	○ ○ ○ ○
		3/3	14	5	○ ○ ○ ○
	Pooled	3/3	12	5	○ ○ ○ ○
		3/3	13	5	○ ○ ○ ○

Year	Strain	Egg-material			Mice (intracerebral inoculation)
		Number of eggs	Number of passages	Harvesting time (days)	
1957	Yokoo	3/3	8	5	● ● ● ● ● 5 8 8 10 10 ↓ ● ● ● ● ● 5 5 5 5 5
	Iwao	3/3	5	5	△ ● ● ● ● 1 8 9 9 9
		3/3	6	5	● ● ● ● ● 5 5 5 5 5
	Hasegawa	3/3	5	5	● ● ● ● ● 12 12 12 12 12 ↓ ● ● ● ● ● 6 6 7 7 8
	Control††	3/3	5	5	○ ○ ○ ○ ○ ↓ ○ ○ ○ ○ ○
	Kobayashi	3/3	8	5	● ● ● ● ○ 7 7 8 8
		3/3	10	5	● ● ● ● ● 5 5 7 8 8
	Midori	3/3	8	5	● ● ● ● ● 4 4 5 5 11
		3/3	10	5	● ● ● ● ● 4 4 5 5 5
	Sakurai	3/3	5	5	○ ○ ○ ○
		3/3	6	5	○ ○ ○ ○
		3/3	7	5	○ ○ ○ ○
	Yamasaki	3/3	5	5	○ ○ ○ ○ ○
		2/3	7	5	○ ○ ○ ○ ○

* Numerator: Number removed for mice inoculation.
Denominator: Number inoculated.
△ Accidental death.
○ Normal.
⊘ Equivocal.
● Death with typical symptoms.
Number: Days from inoculation to death or killing.
† Inoculated with 20 times concentrated material.
†† Inoculated with physiological saline in the 1st generation.

5—8 passages on to chick embryo in 1957. Especially in the case of Hasegawa-strain, the control litter mice inoculated with saline-passaged material showed no symptoms, while those inoculated with patient's serum passaged material were attacked in 100 percent.

Changes on chorio-allantoic membrane: No specific lesion was observed in spite of the close every-day examination through the window and on the removed chorio-allantoic membrane. Sometimes thickening, white mudiness, spotted knots were found, but these changes were observed also in the control saline-passaged group. On the whole, symptoms of mice infected with the fixed virus differed not much from that stated in the earlier reports. As the virus became more adapted to the mouse, the severity and frequency of the paresis of legs became more evident. In the moribund stage, conjunctivitis or gum in the corner of the eyes was usually observed.

2. *Identification test of the virus.*

a) The result of neutralization test of Ohki (isolated in 1951) and Watanabe (isolated in 1955) strains with antiserum of Ohki strain is shown in Table 2a: Neutralization indices proved 1,600 against Ohki

Table 2. Neutralization test of the isolated strains with antiserum of Ohki strain

Year	Strain	Serum	LD_{50}	N. I.
a) 1955	Ohki	Ohki	4.0	1600
		normal	7.17	
	Watanabe	Ohki	3.17	400
		normal	5.77	
b) 1957	Hasegawa	Ohki	3.5	500
		normal	6.17	
	Yokoo	Ohki	3.0	100
		normal	5.0	
	Ohki	Ohki	5.0	3200
		normal	6.5	
	Midori	Ohki	3.33	250
		normal	5.77	
	Kobayashi	Ohki	3.67	130
		normal	5.77	
	Iwao	Ohki	2.67	100
		normal	4.67	

N. I.: Neutralization index.

strain and 400 against Watanabe strain. The results of neutralization test of strains isolated in 1957 with antiserum of Ohki strain are shown in Table 2b: Ohki, Yokoo, Hasegawa, Kobayashi, Midori and Iwao strains showed the neutralization indices of 3,200, 100, 500, 130, 250 and 100, respectively.

b) *Complement fixation test* with convalescent or immune serum and the antigens from Ohki (1951), Watanabe (1955), Yokoo and Hasegawa (1957) strains. As shown in Table 3, complement fixation test with convalescent sera or antiserum of Ohki strain and antigen from Watanabe

Table 3. Complement fixation test of measles convalescent sera with antigen from the mouse-fixed virus

Convalescent serum	Days from onset to sampling	Antigen	Dilution of Serum						Controls*)	
			1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	Serum	Antigen
Takeda	66	Watanabe	4	4	4	4	3	0	0	0
		Ohki	4	4	3	2	1	1	0	0
Kobayashi	112	Watanabe	4	4	4	4	4	1	0	0
		Ohki	3	3	3	3	1	1	0	0
Standard-Anti-Ohki-G. P. Serum		Watanabe	4	3	1	0	0	0	0	0
		Ohki	4	4	2	0	0	0	0	0
Koyama	9	Watanabe	0	0	0	0	0	0	0	0
	22		0	0	0	0	0	0	0	0
	64		2	1	0	0	0	0	0	0
	93		3	3	3	2	0	0	0	0
	120		1	0	0	0	0	0	0	0
Mayuzumi	3	Watanabe	0	0	0	0	0	0	0	0
	24		0	0	0	0	0	0	0	0
	68		4	4	3	2	0	0	0	0
	135		2	1	0	0	0	0	0	0
Yokoo	1	Yokoo	0	0	0	0	0	0	0	0
	27		4	4	2	2	1	0	0	0
	88		4	4	4	2	0	0	0	0
	112		0	0	0	0	0	0	0	0
Kitamura	3	Hasegawa	0	0	0	0	0	0	0	0
	34		4	3	2	0	0	0	0	0
	63		4	4	4	3	1	0	0	0
	130		0	0	0	0	0	0	0	0

G. P.: Guinea pig.

Numbers 4, 3, 2, 1 and 0 indicate 100, 75, 50, 25 and 0% inhibition of hemolysis respectively.

* Dilution 1 : 4.

or Ohki strain proved positive. Complement fixation test of 4 patients' convalescent sera taken successively 4 to 5 times with the lapse of days using antigen from Watanabe, Yokoo and Hasegawa strains, proved that all the sera taken at the initial stage of the disease were negative, but changed to positive 27 days after onset and then gradually turned to negative again, with the exception of the sera from patient Mayuzumi, which showed 1 : 32 positive on the 68th day and 1 : 4 positive still on the 135th day.

c) *Neutralization test with convalescent sera* and Hasegawa- or Yokoo-strain.

The results of neutralization test of the two patients' convalescent sera taken several times following the days are shown in Table 4. The

Table 4. Neutralization test with Yokoo and Hasegawa strains isolated in 1957 against measles convalescent sera

Strain	Days from onset to sampling	LD_{50}	N. I.
Yokoo	7	0	3
	40	3.66	70
	control	5.5	—
Hasegawa	5	4.5	10
	58	3.5	100
	91	3.66	70
	control	5.5	—

N. I.: Neutralization index.

neutralization index was under 10 on the 5—7th day, 70—100 on the 40—50th day and 70 even on the 91st day.

Discussion

In 1948, *Arakawa* succeeded in adaptation of the virus to young mice using patient's serum taken within 24 hours after first symptoms. In the present experiment, however, sera taken at 24—48 hours after onset were used, and consequently the result was not satisfactory as in the case of preceding experiment.

Anderson and *Goldberger* (17) were the first to succeed in the transmission of virus to monkey. Since then *Nicolle* and *Conseil* (18) marked the time for bleeding at the stage within 24 hours before the appearance of *Koplik's* spots or within 36 hours after exanthema. *Wada* (5) was successful in the fixation of the virus to mouse with the serum taken at

the stage of *Koplik's* spots. These facts correspond with the results of *Peebles* et al. (19), who observed experimentally that the period of viraemia continued 2—5 days. From our results, however, it may be concluded that the period in which the virus concentration in blood is highest is at its initial stage. It has been already (20) proved that the mouse-adapted measles virus has no relation to any spontaneous mouse virus, at least to the GD VII strain of encephalomyelitis virus or to ectromelia virus. In the present isolation experiment of Hasegawa strain, saline passaged control was set up in chick embryo cultivation, and inoculated with this control material to mice of the same litter as used for inoculation of the patient's serum passaged material. Then, all the mice were attacked by the latter material, but all the control mice inoculated with saline-passaged material remained unattacked. From this, it may well be concluded that there occurred no contamination of spontaneous viruses neither in mice nor in fertile eggs.

In addition to the immuno-serological identification, the successful re-transmission of the virus to human beings (3) or monkeys (21, 22), therapeutic or prophylactic efficacy of gamma globulin of hyperimmune horse sera (23, 24), and the prophylactic effect of vaccine (24, 25), all support our contention that the mouse-adapted virus is of measles origin.

Moreover, *Suzuki* et al. (7) reported the agreement of their mouse-adapted strains with *Arakawa's* Ohki and Hasegawa strains and with Edmonston strain of Dr. *Enders*, from the point of complement fixation test. They reported, in addition, that these strains showed the same behaviour in tissue cultures.

Conclusion

1. The measles virus can be isolated and fixed to mouse after 5 to 8 serial passages on to the chorio-allantoic membrane of developing chick embryo. Such results were obtained with one strain in 1955, and with five strains in 1957. No virus was isolated by intracerebral inoculation with control saline-passaged chick embryo material to mice of the same litter as used for inoculation of the patient's serum passaged material.

2. The newly isolated strains showed common antigenicity with Ohki strain isolated and identified in 1951 by virus neutralization and complement fixation tests.

3. Sera taken from patients with the lapse of days, showed the rise and fall of neutralizing and complement fixing antibodies against the mouse-adapted measles virus.

This study was aided in part by grant from the Ministry of Education in 1956 and from Japan Waksman Foundation in 1958.

The authors wish to acknowledge Professor Hidetake *Yaoi*, Director of Department of Bacteriology, Yokohama University School of Medicine, for his kind advices and Professor Tomoichiro *Akiba*, Director of Department of Bacteriology, Medical Faculty of the University of Tokyo for his encouragement.

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