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A CASE OF RABIES WITH A PROLONGED INCUBATION PERIOD AND AN UNUSUAL VARIATION PHENOMENON OF THE VIRUS ISOLATED THEREFROM

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We have isolated a virus strain from the brain of a patient who died of rabies in January 1948 following a very long incubation period. We are now continuing immunological studies with this strain. In the course of experiments with mice, a curious variation phenomenon was noted, which seems to us to be a sufficient interest to merit description and discussion.

Case record and the isolation of virus

On July 15, 1945, a girl (■■■■), 6 years old, was bitten on her right cheek by a mad dog in a street near her home in ■■■■■, Tokyo. Prophylactic treatment with antirabic vaccine was not administered. With the usual treatment for wounds, she recovered perfectly within 14 days and was well for about two and half years. Suddenly, on January 12, 1948, exactly 911 days after the bite, she became ill with the typical symptoms of rabies and died on Jan. 14. Autopsy was performed in the Department of Pathology of this university 34 hours after death.

For the purpose of isolating the virus, sterile bits of Ammon's horn and Substantia nigra were emulsified with sterile sand in nutrient broth (pH 7.6) to make a 10 per cent suspension, which was centrifuged at 3,000 r.p.m. for 15 minutes; 0.02 cc of this supernatant was then injected intracerebrally into 5 mice (all mice employed in this study were 8~12 g in weight) and 0.1 cc into a rabbit (No. 1). As prescribed by Webster and Dawson (1), two mice were sacrificed on the 5th and 7th days respectively in order to examine for Negri bodies; the remaining three mice and rabbit

No. 1 were watched for signs of rabies. All of the latter 3 mice became ill on the 10th and 11th days, showing typical rabic symptoms such as severe paralysis and prostration.

While rabbit No. 1 showed no signs of illness for 30 days, another rabbit (No. 2), which had been injected intracerebrally with a mixed suspension of the medulla and pons of the patient's brain at the same time when rabbit No. 1 was injected, died on the 6th day with typical rabic symptoms. The brain suspension of rabbit No. 2 was stored in a refrigerator and then injected intracerebrally into rabbit No. 1 on the 31st day. The latter revealed rabic symptoms and died 6 days later. The development of rabies in rabbit No. 1 seems, however, due to the virus which existed in the human material from the first injection, the reason for this being that incubation of rabbit brain passages carried out thereafter always took longer than 10 days as will be mentioned in the next paragraph.

Bacterial culture throughout these procedures were always negative.

The characteristics and the identification of isolated virus (Takahashi strain)

The virus strain (Takahashi) isolated from mouse and rabbit brains has been maintained in our laboratory by passage through mouse and rabbit brains respectively. The mouse passages are being continued up to the 35th generation; its average incubation is 7.13 ± 1.49 days (233 mice) and the mean length of life is 7.70 ± 1.48 days for 10 per cent brain suspensions. Symptoms of infected mice are typical of rabic encephalitis and no remarkable differences have been observed from those attributable to fixed virus strains. The rabbit passages are now in the 15th generation and no animal has died less than 10 days after inoculation. Rabbits show typical paralysis and salivation following symptoms of excitement and irritation and die within one to three days after the first symptoms; it should be pointed out that all animals had no attack of fever.

Negri bodies in the brain of mice and rabbits examined at various passage generations, as well as in the brain of a guinea pig, which had been inoculated intracerebrally with the brain suspension of rabbit No. 2 and which had died on the 16th day, were all positive by histological examination.* It should be mentioned, moreover, that Negri bodies were also found in the brains of two first generation mice sacrificed five and seven days after the injection respectively. This seems to indorse the report of Webster and Dawson (1) concerning early diagnosis of rabies by mouse inoculation.

Infectivity tests for mice of the Takahashi strain were carried out through various inoculation routes, representative results of them being summarized in Table 1. Suspensions to be injected were prepared by nutrient broth (pH 7.6); the supernatant

* The authors are indebted to Dr. T. Okonogi of the Department of Pathology, Keio Gijuku University School of Medicine, for assistance in this examination.

Table 1. Infectivity in mice of the Takahashi strain.

Inoculation routes	Generation of mouse passages	Dilution of virus suspension							LD ₅₀
		10 ^{-0.7}	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
Intracerebral ⁽¹⁾ (0.02 cc)	II		X, 10, 13	11, 24, S	X, 11, 15	X, S, S	S, S, S	X, S, S	3.25 ⁽²⁾
	III			X, 9	14, 15	17, 19	17, S		5.00
	IV		11, 11, 13	10, 15, 16	X, X, S	X, 24, S	S, S, S	S, S, S	3.00
Subcutaneous (0.3 cc)	IV		1/5 ⁽²⁾	1/5	0/5	0/5			<1.00
Intraperitoneal (0.3 cc)	"		1/5	1/5	0/5	0/5			<1.00
Intravenous (0.1 cc)	"		2/4	1/5	0/5	0/5			1.22
Intrathoracal	"	3/3	3/3	1/3	0/3	0/3			1.75
Intramuscular (0.02 cc)	XV	3/3	3/3	2/3	2/3				3.00

⁽¹⁾ Each numeral indicates duration of life of a mouse killed by rabies.

X = Discarded mice (killed by other causes than rabies).

S = Survived mice.

⁽²⁾ 1/5 = One out of five mice inoculated died of rabies.

Intramuscular inoculation was performed into M. gastrocnemius.

⁽³⁾ Expressed in logarithm of dilution with minus sign omitted.

centrifuged at 1500 r.p.m. for 5 minutes was diluted with the same broth. It seems noteworthy that LD₅₀ (Reed and Muench) of the intracerebral inoculation is somewhat irregular and has far lower dilutions than that for fixed virus (Nishigahara strain*), the latter's LD₅₀ being a dilution of 10⁻⁶ at frequent examination. Infective titres by peripheral routes were generally low except in the intramuscular one; but it seems to correspond with experiments by Kimura (2) with fixed virus that relatively more animals were killed by intrathoracal inoculations.

Table 2. Neutralization tests between the Takahashi and Nishigahara (fixed virus) strains.

Rabbit immune serum	Dilution of virus in mixtures with serum ⁽¹⁾									
	Takahashi strain					Nishigahara strain				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Takahashi	1/3	0/3	0/3	0/3		3/3	0/3	0/3	0/3	0/3
Nishigahara	0/3	0/3	0/3	0/3			0/3	0/3	0/3	0/3
Control ⁽²⁾	3/3	3/3	2/3	2/3					3/3	2/3

⁽¹⁾ Each dilution of virus mixed with an equal volume (0.2 cc) of the serum was injected intracerebrally in mice after standing at 37°C for 2 hours.

⁽²⁾ "Control" serum was prepared by injecting with normal mouse brain in a rabbit.

1/3 = One out of three mice inoculated died of rabies.

* Thank are due Dr. K. Ando of the National Institute of Health of Japan for delivery to us of this strain.

Neutralization tests were practised crosswise between the Takahashi and Nishigahara strains with rabbit immune sera of both (Table 2). Undoubtedly, the Takahashi strain seems to have the same antigenic characteristics as fixed virus. Moreover, mice immunized actively by intraperitoneal inoculations with live Takahashi virus resisted strongly against the intracerebral infection with the Nishigahara virus.

An unusual variation phenomenon of the Takahashi strain

During experiments in mice with the active immunization of the Takahashi strain, a variant strain was unexpectedly obtained. When 30 mice had been inoculated intraperitoneally first with 0.5 cc of an 1 per cent suspension of the Takahashi strain (a mixture of two mouse brains at the 4th generation of passages), and then, 4 days later, with 0.1 cc of a 10 per cent suspension of the mixed brains of 3 other mice of the same generation for the purpose of attaining active immunization, 17 mice suc-

Fig. 1. Animal passages of the Taka-men strain.

All inoculations were done intracerebrally with a 10 per cent brain suspension.

Each numeral indicates the duration of life of the animals.

Mice in the first row were inoculated with the Takahashi strain intraperitoneally (one group of 30 mice).

⁽¹⁾ Died without confirmation of rabic symptoms.

S = Survived.

cumbed to typical rabic symptoms after the following incubation periods (from the first inoculation): 2 mice on the 5th day, 6 on the 6th day, and one mouse each on the 7th, 8th, and 15th days respectively. We have tried then to inoculate a brain suspension of one of the mice which died on the 6th day to another healthy mice intracerebrally and the virus has been passed in mice thereafter up to the 57th generation.

As shown in Fig. 1, this virus strain kills mice constantly within shorter periods than the Takahashi strain; its average incubation is 4.62 ± 0.60 days (208 mice) and the mean length of life is 5.22 ± 0.59 days. Intracerebral inoculations in rabbits manifest, moreover, far shorter incubation periods than for the Takahashi strain. This is revealed in Fig. 1. Calling this probable variated virus, the "Taka-men" strain, we have continued further investigations.

The characteristics of the variant, "Taka-men" strain

Although no remarkable differences in the symptomatology between mice infected with the Takahashi and Taka-men strains exist, the irritability is relatively lower and the paralysis of the hind legs seems more remarkable in mice infected with the latter strain. Rabbits inoculated with the Taka-men strain also show a lower irritability and more marked paralysis, but a noticeable difference is in the constant fever attacks ($40^{\circ} \sim 41^{\circ}\text{C}$) observed simultaneously with initial hind leg paralysis, which can never be observed in infection with the Takahashi strain.

As a result of infectivity tests with the Taka-men strain, it has become clear that its intracerebral titre for mice is far higher than that of the Takahashi strain, and nearly the same as that of the Nishigahara strain (Table 3). As shown in Table 3, the intracerebral titre for mice of the brain suspension of infected mice did not

Table 3. Infectivity in mice of the Taka-men strain.

Inoculation routes	Dilution of virus suspension								LD ₅₀
	10 ^{-0.7}	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
Intracerebral (0.02 cc)	I ⁽¹⁾	4, 4, 7	6, 7, 7	7, 7, 7	6, 6, 6	6, 6, 6	5, 7, S	X, 27, S	6.60
	II ⁽²⁾		7, 7, 7	5, 6, 7	7, 7, 7	7, 7, S	X, 7, 19	S, S, S	6.25
Subcutaneous (0.3 cc)	3/3	3/3	0/3	0/3					1.50
Intraperitoneal (0.3 cc)	2/2	2/2	3/3	1/3					2.75
Intrathoracal (0.1 cc)	3/3	3/3	1/2	0/3					2.00
Intramuscular (0.02 cc)	3/3	3/3	1/3	0/3	0/3				1.75

See footnotes to Table 1.

⁽¹⁾ Suspension was prepared from mouse brain (of the 9th generation).

⁽²⁾ Suspension was prepared from rabbit brain (of the 1st generation).

differ from that of an infected rabbit, and this was the same for the Takahashi strain, its LD₅₀ of the brain suspension of an infected rabbit being a dilution of 10^{-3.75}.

Table 4 shows neutralization tests for the Taka-men, Takahashi, and Nishigahara strains and also Japanese encephalitis virus (Kalinina strain*). It seems obvious that the Taka-men strain shows no changes in its antigenic character from the original Takahashi strain.

Table 4. Neutralization tests among the Taka-men, Takahashi, Nishigahara strains, etc.
(a)

Rabbit immune serum	Dilution of virus in mixtures with serum											
	Taka-men strain							Takahashi strain				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Taka-men.....	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Takahashi	3/3	0/3	0/3	0/3	0/3	0/3	0/3					
Nishigahara.....	0/3	0/3	0/3	0/3	0/3	0/3	0/3					
Control			3/3	3/3	2/3	1/3	1/3	3/3	3/3	1/3	1/3	

(b)

Rabbit immune serum	Dilution of virus in mixtures with serum											
	Nishigahara strain						Kalinina strain ⁽¹⁾					
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Taka-men.....	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3
Takahashi												
Nishigahara.....												
Control			3/3	3/3	1/3	1/3	3/3	3/3	3/3	1/3	1/3	0/3

See footnotes to Table 2.

⁽¹⁾ Japanese encephalitis virus.

Negri bodies in the brain of mice infected with the Taka-men strain have been found positively by occasional examinations up to the 38th generation. Those in the rabbit brain were also positive in the 1st and 3rd generations. The negrigenesis of this strain in later generations is being pursued now.

Filtrability

Both Takahashi and Taka-men strains have been found to pass the Berkefeld N and V types of filter, but not the Seitz E. K. paper filter. The filtration tests were conducted after a preliminary treatment of the filters by 10% horse serum broth (pH 7.6) and each material to be filtered consisted of 20 cc of an 1% suspension in the same broth of the infected mouse or rabbit brain. Bacterial contamination was always negative.

* Thanks are again due Dr. K. Ando for delivery to us of this strain.

DISCUSSION

The Takahashi strain, undoubtedly, belongs to the rabies "street" virus according to results stated above. And the possibility that the girl, from whom the virus was isolated, had been attacked with rabies from the dog bite 911 days earlier seems to be accepted as likely, because she had had no opportunity of being bitten by or of contact with dogs, cats or other animals during the incubation so far as we know. Although the symptoms of the dog are not exactly known, it has been testified by her family and by neighbours that some other children had also been bitten at that time.

A prolonged incubation period of rabies, such as of 911 days, is very rare. It must be noted that the victim, a 6 years old girl, was bitten on her face and no antirabic vaccination was enforced. The incubation period which was longer than two years is a very rare case according to literature (3). Moreover, the activating moment of "latent" virus in vivo, as described by Rochaix (4), Stuart and Krokorian (5), etc., were not noticed in the case reported here.

The Taka-men strain isolated from the brain of a mouse inoculated intraperitoneally with the Takahashi strain seems to be a rabies virus according to serological and other examinations; it seems to have varied abruptly from the original strain. It differs serologically from the Japanese encephalitis virus and differs distinctly in symptomatology and virulence from the mouse encephalomyelitis virus (Theiler).

The principal differences between the Takahashi and Taka-men strains are the short incubation periods in mice and rabbits, the rise in intracerebral infectivity in mice, and pyrexia in rabbits caused by the latter strain. These changes seem to show a tendency to "fixation", though not perfectly proved. In this respect, we tried to measure the infective titre in rabbits (2,000~2,500 g in weight) of the two

Table 5. Infectivity in rabbits.

Inoculation routes	Dilution of virus suspension														
	Takahashi strain							Taka-men strain							
	10 ^{-0.7}	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ^{-0.7}	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Intracerebral (0.1 cc)		13		14	6 ⁽¹⁾	16	X		5	6		6	5 ⁽¹⁾	S	S
Subcutaneous (0.5 cc)	50	30	29					S	S	S	S				

Each numeral indicates duration of life of a rabbit killed by rabies.

⁽¹⁾ Showed no typical symptom but the virus was isolated from the brain by the transfer to mice.

X=Died on the third day without rabic symptoms, and the virus was not isolated from the brain.

S=Survived.

strains by the intracerebral as well as the subcutaneous routes (Table 5), and have observed, in a small number of animals, a fall of the peripheral infectivity of the Taka-men strain in rabbits.

Although Negri bodies have been produced by the infection with the Taka-men strain, there are many reports (3, 6, 7) indicating their production by fixed virus. Further studies should be required on this subject.

The Taka-men strain seems, therefore, to be a variant that arose abruptly from the original Takahashi strain. We cannot find any report that such variation of the rabies virus originated from the intraperitoneal inoculation to mice; the phenomenon reported here should be recognized as unusual for this reason. We could discover no similar phenomenon in two repeated experiments conducted under similar conditions with 30 mice and the Takahashi strain of the 8th and 11th mouse generations. Although there are a few reports (8, 9) relative to the obtainment of two different strains from one "street" virus, they all have been observed by the intracerebral passages in rabbits.

SUMMARY

A case of human rabies has been reported here which seems to have had the extra long incubation period of 911 days: the victim was a 6 years old girl bitten on her face by dog.

A virus strain of the rabies has been isolated from the patient's brain and an unusual variation phenomenon has been observed during experiments with mice. This variation was an abrupt change which seemed to show a tendency to virus "fixation" and was noted in the intraperitoneal inoculation of mice.

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