Avianized Rabies Virus Infection in Mice

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ABSTRACT. Weanling mice were infected with avianized H virus of low pathogenicity, and various virological and immunological parameters were examined. In comparative study with other strains, mice infected with F and Str. viruses showed very high virus multiplication in the brain and all the infected mice died. In mice infected with H virus, the virus multiplied and, then, disappeared rapidly in the brain, and the majority of mice survived. CF antigen could be demonstrated in the brain of mice infected with H virus, even after the disappearance of the virus. In mice infected with Fl.H virus, the virus multiplied at such a low level that it was difficult to detect a distinct CF antigen from these mice. Mice infected with H and Fl.H viruses produced high virus-neutralizing antibody and CF antibody in serum. When a higher dose of H virus in the inoculum was employed, the virus multiplied earlier and disappeared earlier in the mouse brain compared with those infected with lower doses. In addition, in these mice, neutralizing antibody were produced earlier in serum. Among mice inoculated with a low dose of H virus, there was a decrease in the incidence of infection, and the onset of illness was delayed. However, their death rate was higher than that of those infected with high doses. No virus was demonstrated in the brain of the dead mice. In the early stage of H virus infection, no inhibitors or incomplete virus particles were found in the brain. When various types of antigen harvested from the brains of mice infected with H virus were examined, the protective ability or ability to produce neutralizing antibody in serum was correlated with the virus titers, and the ability to produce CF antibody in serum was correlated with the CF antigen titer in the brain. The disappearance of the virus from mouse brains infected with H virus in early stage, recovery from clinical illness and the rapid development of virus neutralizing antibody were discussed.—**KEY WORDS:** chick embryo passage, mouse pathogenicity, rabies.

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Street (Str.) or fixed (F) rabies virus substantially decreased their pathogenicity for mammals after serial passage in chick embryo [7, 8, 12]. The weanling mice survived with few clinical signs after intracerebral inoculation with the high chick embryo passage virus (Fl.H) of Flury strain [8]. The results of a series of investigations carried out to understand this phenomenon, suggested that some immune mechanisms are involved [3, 6, 9, 10, 14]. Few studies have been done to analyze the mechanisms of the phenomenon involving the avianized rabies virus of the Nishigahara strain [13].

The present experiments were designed to clarify the characteristics of infection of mice with the high chick embryo passage

rabies virus of the Nishigahara strain and to provide data which related to the mechanisms of clearance of virus and survival after infection with this virus. Comparative studies with other strains (F, Str. and Fl.H virus) were carried out in mice prior to main experiments.

MATERIALS AND METHODS

Mice: Three to four-week-old ddF male mice were used.

Embryonated eggs: Fertilized eggs laid by white Leghorn were purchased from a commercial source.

Viruses: Str., F, H and Fl.H viruses were used [11]. Str. virus was street virus origin-

ated from the brain of a dog naturally infected with rabies. Mouse brains infected with Str. virus were used in the present experiment. F virus was a rabbit fixed virus of the Nishigahara strain, and passaged more than 30 times in mice. H virus was a high chick embryo (CE) passage (>285th) virus of the Nishigahara strain, which demonstrated extremely low pathogenicity in mice after the intracerebral inoculation [12]. Fl.H virus was a high CE passage (>235th) virus of the Flury strain with virtual non-pathogenicity in mice [8].

Estimation of virus titers: Three-week-old ddF male mice were used for the titration of Str. and F viruses. Seven-day-old eggs were used for the titration of H and Fl.H viruses [13]. Serial tenfold dilutions were prepared from the virus specimen with bouillon (pH 7.2). Mice were inoculated intracerebrally with 0.03 ml of each dilution. Unless otherwise indicated, a 50% lethal dose (LD_{50}) was expressed with $\log_{10}/0.03$ ml. Seven 7-day-old eggs were inoculated with 0.1 ml of each dilution. Unless otherwise indicated, a 50% egg infective dose (EID_{50}) was expressed with $\log_{10}/0.1$ ml.

Virus neutralization test: The conventional method was used as follows. Serial tenfold dilutions of F virus were prepared with bouillon (pH 7.2). To each dilution was added a threefold dilution of test serum or control normal serum inactivated by heating at 60°C for 30 minutes. The mixture was incubated at 37°C for one hour. Four 3-week-old mice were intracerebrally inoculated with 0.03 ml of each mixture to estimate the infective titer. The neutralization titer of the test serum was expressed as $\log_{10}$ differences in the infective titers between the test and control sera.

Detection of CF antigen and estimation of its titer: Emulsions were prepared in PBS, pH 7.2, from brains of mice or chick embryos by the method previously described [11], and was dried in a desiccator

containing calcium chloride at 15–25°C under vacuum (20–40 mm/Hg) for 2–3 days. The dried antigen was resuspended in distilled water and frozen at –20°C. Complement fixation (CF) antigen was determined by the TRC (titration of residual complement) method [11], and was expressed with the fixation titer (FT) of the undiluted original suspension (10%). In some cases, serial twofold dilutions was prepared from the original suspension to estimate the amount of antigen, and antigen dilution of FT20 (AgD_{20}) was calculated [11].

Detection of CF antibody: By the TRC-method, CF antibody was detected in the same manner as CF antigen. In this method, the mixture of the negative antigen (NA) series contained 0.1 ml of test serum dilution (1:2), 0.2 ml of control antigen dilution (1:40), and 0.1 ml of complement dilution (1:8). The mixture of the positive antigen (PA) series had the same composition as that of the NA series, except that the control antigen dilution was replaced by PA dilution. The mixture was left overnight at 5°C. Residual complement was then estimated, and FT was calculated. Antigen solutions were prepared from the brain of a goat infected with F virus (PA) by the same method as used for preparation of the test antigen solution. The control antigen was prepared in a similar manner from an uninfected goat. They were freeze-dried and stored at –20°C.

Mouse infection with viruses of high and low virulence: The tests were performed with Str., F, H and Fl.H viruses to compare rabies viruses of high virulence with those of low virulence. As shown in Fig. 1, 4-week-old mice were inoculated intracerebrally with 0.03 ml of each virus. Groups of 5 mice each were sacrificed at a given interval of time up to 30 days after inoculation. The brain and blood were separately harvested from each mouse. The brains and blood collected from the 5 mice of each group

were pooled separately. The pooled brains were used for the estimation of the virus titers and CF antigen. Sera obtained from the pooled blood were used for the estimation of CF and neutralizing antibody titers. The titers were compared with clinical symptoms.

Viral antigens in the brain of mice infected with H virus: Two hundred 4-week-old mice were intracerebrally inoculated with 0.03 ml of 10^{-4} dilution of the stock H virus ($10^{6.0}\text{EID}_{50}$). As shown in Table 1, groups of 30 mice each were sacrificed at given intervals up to 20 days after inoculation. Brain and blood were collected and pooled for each group. The brain pools were examined for amounts of virus and CF antigens. Sera were examined for titers of neutralizing and CF antibodies. From the pooled brains, 10% extracts were prepared by adding the PBS (pH 7.2) in 9 ml per g of the tissue, homogenizing the mixture and separating the supernatant by centrifugation. The virus was inactivated by uv irradiation. Groups of 20 mice each were inoculated intraperitoneally with 0.3 ml of the inactivated fluid six times every other day. Ten mice of each group were challenged by intracerebral inoculation with 100–500 LD_{50} of F virus 7 days after the last inoculation. Blood samples were collected from the remaining ten mice in each group. Neutralizing and CF antibody titers were estimated in the pooled serum by the conventional methods.

Immunizing effect of H virus: Three dilutions, 10^{-1} , 10^{-3} and 10^{-5} , of H virus ($10^{7.0}\text{EID}_{50}$) were intracerebrally inoculated into groups of approximately 83 four-week-old mice each. Five mice selected at random in each group were sacrificed at times indicated in Fig. 2., and brains and blood were pooled. Virus titers were estimated in the pooled brain, and neutralizing antibody titers in the sera were determined. The remaining mice were observed daily for

clinical symptoms. The mice inoculated with H virus were challenged by intracerebral inoculation with 100–200 LD_{50} of F virus 21 days after inoculation. Those mice which withstood the challenge inoculation were regarded as mice infected with H virus. Mice manifesting symptoms at the time of challenge were regarded as infected ones and held under observation without being challenged.

Assay of a substance inhibiting virus multiplication: Thirty 3-week-old mice were intracerebrally inoculated with 0.03 ml of 10^{-1} dilution of H virus ($10^{6.7}\text{EID}_{50}$). They were sacrificed 5 days later to collect the brain and blood. A three fold emulsion was prepared from the brain and centrifuged. The supernatant was inactivated by uv irradiation. Sera were diluted threefold. As a control, a threefold dilution was prepared from the sera of normal mice. Three groups of 40 four-week-old mice each were intracerebrally inoculated with 0.03 ml of the brain, serum samples, or the control serum dilutions. As shown in Table 4, the 40 mice of each group were divided into four subgroups of ten each. The ten mice of each subgroup were intracerebrally inoculated with each of tenfold dilutions of H virus 24 hours later. They were held under observation for 14 days, and then challenged by intracerebral inoculation with 100–200 LD_{50} of F virus. Those which survived were regarded as mice infected with H virus. In each subgroup, the infective titer (ID_{50}) of H virus was calculated. The difference in this titer between the experimental and the control group was considered as the virus multiplication-inhibiting or the infection-preventing titer.

RESULTS

Detection of virus, CF antigen and CF and neutralizing antibodies in mice infected with viruses of high and low virulence: F virus

Fig. 1. Detection of virus, CF antigen and CF and neutralizing (VN) antibody in mice infected with rabies viruses of high and low virulence.

Table 1. Demonstration of various immune substances in the brain of mice infected with H virus

Days after inoculation	In brain						In serum	
	Virus titer (EID ₅₀)	CF antigen (FT)	Protective ability (%)	VN antibody productive ability (VN titer)	CF antibody productive FT	ability liter	VN antibody (titer)	CF antibody (FT)
3	5.2	52	30 ^{a)}	3.1	51	1: 4	1.0	0
5	5.7	81	90	5.2	100	1:16	0.8	0
7	4.5	92	90	5.1	100	1:16	2.0	0
10	2.5	72	56	3.6	100	1:32	2.5	0
15	0.5	63	0 ^{a)}	1.2	100	1:16	3.0	91
20	0.5	56	0 ^{a)}	0.7	0	1: 2	4.1	73

a) The interval between inoculation and death was a little longer (0.6–1.0 days).

strain caused the most severe infection among all the viruses (Fig. 1). Mice inoculated with F virus began to manifest symptoms on 4 days and died within 7 days after inoculation. The intracerebral virus titer (log₁₀) began to increase rapidly from the 2nd day and reached a plateau level of 6.8–7.4 LD₅₀, 4 days after inoculation. CF antigen, FT reached 100 as early as 3 days after inoculation, and AgD20 attained the maximum of 5 (1:320) 5 and 6 days after inoculation. Neither CF nor neutralizing antibody could be detected in the serum. When mice were inoculated with Str. virus, clinical infection was observed in about 7 days after inoculation. All the mice died within 11 days after inoculation. intracerebral virus titer reached 5.4 LD₅₀ seven days after inoculation, remaining at a level of 5.0–5.5 LD₅₀ later. CF antigen titer (FT) increased rapidly reaching 100 as early as 5 days after inoculation, and AgD20 reached a maximum, or 5 (1:320), 9 days after inoculation. A slight increase was observed in serum neutralizing antibody titer after the lapse of 7 days. No CF antibody was detected after inoculation. H virus induced a substantially milder infection in mice than did Str. or F virus. A majority of the mice demonstrated only mild symptoms over a period of 6 to 16 days with few deaths. In mice infected with H virus, the virus multi-

plication was considerably rapid in the brain. The virus titer reached the maximum 5 days after inoculation followed by a rapid decrease, and reached nil 16 days after inoculation. The appearance of CF antigen was a little delayed compared with the virus appearance. Its titer began to increase rapidly about 5 days after inoculation and showed a high level over a period when the virus titer was decreasing gradually, FT was 100 at seven and ten days and 98 thirteen days after inoculation. Furthermore, the CF antigen could be detected for a long time even after no virus was demonstrated. Neutralizing antibody appeared in serum about 10 days after inoculation and increased thereafter. CF antibody appeared 13 days after inoculation, a little later than neutralizing antibody did. Fl.H virus induced the mildest infection. No infected mice died, but a few of them manifested very mild transient symptoms over a period of 6 to 10 days after inoculation. Patterns of infective virus titers and CF antigen in the brain and neutralizing and CF antibodies in serum resembled those observed in mice inoculated with H virus. However, Fl.H virus generally showed less distinct response than H virus did.

Immunogenic antigens in the brain of mice infected with H virus: When responses in various parameters of the mice inoculated

Fig. 2. Relationship among the dose of inoculation of H virus ($EID_{50}:7.0$) and virus multiplication and neutralizing antibody production.

Table 2. Relationship between the inoculum size and the disappearance of virus in mice brain infected with H virus

	Virus concentration of inoculum		
	10^{-1}	10^{-3}	10^{-5}
Days of illness when virus appeared ($EID_{50}:1.0$ or over)	1	1	2
Days of illness when virus disappeared ($EID_{50}:1.1-1.5$)	7	10	15
Duration of the presence of virus ($EID_{50}:3.0$ or over)	5	7	8
Days of illness when neutralizing antibody appeared (Titer:1.0 or over)	3	5	7
Interval of time between the appearance of neutralizing antibody and the disappearance of virus	4	5	8

with H virus were examined, the results similar to those obtained in the previous experiment (Table 1) were observed. When the ability of the brain to prevent infection (or the rate of prevention) was examined, a very high rate of prevention was exhibited by the emulsions of the brains collected 5 or 7 days after H virus inoculation. The ability of the brain to produce neutralizing antibody was the highest when harvested 5 or 7 days after inoculation. The antigen capable of provoking CF antibody was produced

abundantly in the brain over a period from 5 to 15 days after inoculation. This agrees quite well with the production of CF antigen.

Doses of H virus and the course of infection: The relationship among the dose of inoculation of virus and virus multiplication in the brain and neutralizing antibody production in serum was shown in Fig. 2. The concentration of the virus inoculum was correlated with the rate of virus multiplication and virus disappearance from the brain.

Table 3. Relationship between the dose of inoculation of H virus and death from clinical infection

virus (EID ₅₀ /ml)	Dilution of virus (log)	Illness Inoculated (%)	Infected Inoculated ^{a)} (%)	Died Infected (%)	Intervals between inoculation and death (days)
H (8.0)	-1	24/31(80)	31/31(100)	3/31(10)	7,7,7
	-3	29/35(83)	35/35(100)	5/35(14)	10,10,12,12,15
	-5	23/34(68)	34/34(100)	7/34(21)	12,16,17,17,18,18,18
H ^{b)} (7.7)	-4	11/13(85)	13/13(100)	2/13(15)	12,14
	-5	12/16(75)	16/16(100)	4/16(25)	15,15,19,19
	-6	9/15(60)	11/15(73)	4/11(36)	15,16,18,12
	-7	2/14(14)	2/14(14)	2/2(100)	15,16
	-8	0/16(0)	0/16(0)		
H ^{b)} (8.5)	-0.7	18/20(90)	20/20(100)	2/20(10)	5,10
	-1.2	15/20(75)	20/20(100)	1/20(5)	6
	-3.2	17/20(85)	20/20(100)	2/20(10)	12,14
	-4.8	15/20(75)	20/20(100)	4/20(20)	14,15,15,27
	-5.6	19/20(95)	20/20(100)	2/20(10)	17,21
	-6.4	14/20(70)	15/20(75)	3/15(20)	16,16,20
	-7.0	11/20(55)	12/20(60)	4/12(33)	15,15,16

a) Challenged by intracerebral inoculation with 100–200LD₅₀ of F virus 21 days after inoculation with H virus and survived mice were regarded as mice infected with H virus.

b) Carried out as additional tests.

Thus, in mice inoculated with 10^{-1} dilution of virus, the virus multiplication reached a peak 2 days and declined thereafter until it became undetectable 10 days after inoculation. In mice inoculated with 10^{-3} dilution of virus, the infective virus titer was at a high level over a period of 3 to 7 days after inoculation, began to decline 10 days, and became undetectable 15 days after inoculation. In mice inoculated with 10^{-5} dilution of virus, the virus titer was further delayed, showed a high level over a period of 5 to 7 days after inoculation, and slowly declined. When the production of neutralizing antibody was examined, in the mice with the higher dose of inoculation of virus, the antibody appeared earlier and its titer became higher than in those which received lower amounts of virus. In summary, higher amounts of virus induced earlier appearance of neutralizing antibodies, resulting in earlier disappearance of the virus (Table 2). Table 3 shows the relationship between

doses of H virus and time of death. The main symptoms manifested by the infected mice were depression, ruffled hair coat, and emaciation. No paralysis was exhibited by any mouse clinically infected with the H virus. Some infected mice died after showing the symptoms, while others recovered after a long period of clinical illness. There was a tendency for the mice that received smaller doses to show higher death rates with longer intervals between the inoculation and death. A very low concentration of virus or no virus was detected from dead mice.

Examination of a substance inhibiting virus multiplication in mice infected with H virus: As shown in Table 4, ID₅₀ in each group infected with H virus was 5.4 in the group pretreated with the brain harvested at 5th day of inoculation, 4.9 in the group pretreated with the serum at 5th day of inoculation, and 5.3 in the control group pretreated with normal serum. The results

Table 4. Detection of a substance inhibiting virus multiplication in mice infected with H virus

1st inoculation ^{a)} materials	2nd ^{b)} inoculation virus	No. mice infected/ No. mice inoculated ^{c)} Dilution of H virus (-log)					ID ₅₀ (-log)	Inhibitive value (-log)
		3	4	5	6	7		
5 days brain(1:3) after inoculated with H virus	H	9/ 9	6/ 7	7/ 8	1/ 9	—	5.4	-0.1
5 days serum(1:3) after inoculated with H virus	H	10/10	9/10	5/10	0/10	—	4.9	0.4
Normal serum (1:3)	H	—	9/10	7/10	1/70	0/9	5.3	—

a) Inoculated intracerebrally with 0.03 ml.

b) Inoculated intracerebrally with 0.03 ml of each dilution of H virus 24 hr after first inoculation.

c) Challenged by intracerebral inoculation with 100–200LD₅₀ of F virus 14 days after second inoculation and survived mice were regarded as mice infected with H virus.

indicate little difference in ID₅₀ among the three groups mentioned above. Therefore, none of the materials could inhibit the multiplication of H virus.

Moreover, the brains of mice infected with H virus were examined for the presence of the defective interfering particle (DI particle) of Clark *et al.* [1]. Two virus bands were obtained (data not shown). One band was located at a density of 1.173–1.175, with a EID₅₀ of 5.8 and the other at a density of 1.143–1.153 with a EID₅₀ 4.5. It was impossible, however, to demonstrate the presence of DI particle from either band.

DISCUSSION

Mice were intracerebrally inoculated with strains of virus of different pathogenicity. All the mice inoculated with F and Str. viruses of high pathogenicity manifested typical symptoms and died. On the other hand, of mice inoculated with H or Fl.H viruses of low pathogenicity, only a small number died after clinical infection. When observation was made of the multiplication of these viruses in the mice brain, it is of

interest to note that there was a pronounced difference in behavior between H and Fl.H viruses and F and Str. viruses. H and Fl.H viruses failed to maintain their high virus titers for an appreciable time period.

The CF antigen was detectable in mice infected with H virus for several days even after the disappearance of the virus. This result may indicate that the detection of intracerebral CF antigen is a very reliable method for the diagnosis of rabies virus infection. Chronological examinations were carried out to detect antigen in the brain of a mouse and to quantitate protective capacity of the antigen. The protective ability was not always proportional to its virus titer. A similar tendency was shown in an ability to produce neutralizing antibodies. These abilities were lower at 3rd day of inoculation, when the virus titer was higher, while they were higher at 7th and 10th day of inoculation with lower virus titers. The results suggest the presence of inactivated virus, which might have participated in the development of abilities to prevent infection and to produce neutralizing antibody. The ability to produce CF antibody agreed quite well with CF antigen titers. An exception is

that no antibody was produced in the brain 20 days after inoculation when antigen titer was 56 in this brain. Serum samples collected at such a stage as late as 20 days after inoculation showed high neutralizing and high CF antibody titers. It seems necessary to consider that such high antibody titers may have exerted some effect on the brain sample. Fujisaki [4] reported the presence of neutralizing antibody of considerably high titer in the parenchyma of the mouse brain 2 weeks after intracerebral inoculation with H virus.

H or Fl.H viruses multiply rapidly in the mouse brain and then decline rapidly. To interpret this phenomenon, an experiment was carried out for the possible detection of inhibiting substance for viral multiplication. It was impossible, however, to demonstrate the participation of such a substance in the decrease in virus titers.

In 1972, Wiktor *et al.* [14] indicated that mice inoculated intracerebrally with Fl.H virus produced the neutralizing antibody in serum and brain as early as 2 days after inoculation. Fischman and Strandberg [3] observed that immunosuppressed mice inoculated with Fl.H virus showed high mortality, while immunosuppressed mice which received immune serum almost survived the infection of Fl.H virus. Similar experiments using immunosuppressed or immunodeficient mice were carried out, and it was suggested that neutralizing antibody would participate in the recovery from the infection [6, 9, 10].

When an observation was made on the relationship between the dose of inoculation of H virus and the onset of clinical symptoms, it is of great interest to note that the interval between inoculation and death was prolonged in mice inoculated with a lower concentration of the virus, and that the death rate was higher among these animals. The virus disappeared almost completely

from the animals in the moribund stage. The duration of the presence of the virus before death was longer in mice inoculated with a low concentration of the virus than in those inoculated with a high concentration of the virus. It may have been related with the death rate. On the other hand, neutralizing antibody was produced more rapidly in the blood in mice infected with H virus than in those infected with high virulent F or Str. virus. Besides, the higher virus concentration of the inoculum, the more rapidly the antibody was produced. These results well reflect the early disappearance of the virus in mice inoculated with a high concentration of the H virus.

Koprowski *et al.* [8] observed a phenomenon presenting auto-interfering (AI) death patterns in hamsters or in guinea pigs inoculated intracerebrally with Fl.H virus. These patterns were irregular lethal-dose responses which were not correlated with the dose of virus administered. These authors [8] presumed that an interference might have taken place between the two kinds of rabies virus mixed in the inoculum that a balance might have been lost between the two viruses by dilution of the inoculum. In 1974, Crike and Brown [2] found that DI particles were mixed with the Flury strain of rabies virus in cell cultures. Recently, Clark *et al.* [1] observed that there was no distinct relationship between the presence of DI particles and the pathogenicity of the virus for mice. In the present investigation, an attempt failed to demonstrate the presence of DI particles in the brain of a mouse infected with H virus. It seemed difficult to find DI particles in adult mouse brain infected with rabies or other viruses [5].

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要 約

鶏胚順化狂犬病ウイルスのマウス感染の様相：竹原孝一（日本生物科学研究所）——哺乳動物に対し病原性を減弱した狂犬病ウイルスの鶏胚順化 H 株の離乳マウスにおける感染の様相をしらべた。強毒の固定および街上ウイルスは、著明なウイルスの増殖後に感染マウスは死亡したが、弱毒の H ウイルス感染では、増殖ウイルスは急速に消失し、多くのマウスが耐過生残した。H ウイルス感染マウス脳には、ウイルス消滅後も CF 抗原が証明された。最も弱毒の F1. H ウイルス感染では、ウイルスの増殖は低く、CF 抗原も証明されなかった。H および F1. H ウイルス感染マウスは、血中に高い中和および CF 抗体を産生した。H ウイルスの大量を接種したマウスでは、小量接種マウスに比し、脳中のウイルス増殖およびその消滅は速く、血中中和抗体の産生も速かった。小量の H ウイルスを接種したマウスでは感染率は低く、発病も遅延したが、感染マウスの死亡率は大量接種マウスに比し高かった。しかし、これら死亡マウス脳からウイルスは検出されなかった。H ウイルス感染早期のマウス脳には、ウイルス増殖阻止物質あるいは不完全ウイルス粒子は検出されなかった。H ウイルス感染マウス脳の抗原性、免疫原性を経時的にしらべると、感染防御能と、中和抗体産生能の消長はウイルスのそれと、CF 抗体産生能の消長は CF 抗原のそれと一致した。