Immunohistochemical and Histopathological Study of Experimental Rabies Infection in Mice

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ABSTRACT. An immunohistochemical and histopathological study using the ABC technique was carried out to examine time-sequential virus spread in the central nervous system (CNS) of mice after inoculation with the CVS strain of fixed rabies virus by different routes; intracerebral (ic), intraocular (io), intranasal (in), intramuscular (im) and subcutaneous (sc). Only the ic and io inoculations caused fatal infections, so that detailed analysis was conducted on mice inoculated by these two routes. In ic-inoculated mice, viral antigens were detected mainly in neurons in the cerebral cortex and in the pyramidal cells and granular cells of the hippocampus. After io inoculation, viral antigen was first detected in the trigeminal nerve ganglia, following which it spreads to the cerebral cortex and cerebellum. In the hippocampus only a few cells were viral antigen-positive at the early stage after io inoculation. There were no inflammatory lesions or Negri bodies in the CNS of mice infected by either route. This suggests that clinical signs such as ataxia or depression leading to death may be due to the direct effect of the virus on the functions of neural cells, but not to inflammatory reactions. The ABC method will be useful for the early diagnosis of suspected patients or animals to have the disease when conventional histopathological and immunofluorescent antibody techniques can not detect lesions or viral antigens.—**KEY WORDS:** ABC method, histopathology, intraocular inoculation, monoclonal antibody, rabies virus.

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Rabies virus (RV) causes lethal infections in humans and animals, with the occurrence of acute encephalitis in most cases and paralysis in some cases, the virus being restricted to nervous tissues. The diagnosis of rabies has been based mainly on conventional histopathological examination to demonstrate Negri bodies, since the time that Negri reported these particular intracytoplasmic inclusions [14]. Negri bodies, however, were not detected in any tissues which were found to be positive by virus isolation in mice or in cell cultures [12]. For diagnosis, the current conventional method employs an immunofluorescent antibody (IFA) technique, using fluorescein-conjugated antibodies to detect viral antigen in stamped samples or frozen tissue sections of suspected patients. Under conventional light microscopy, neither inclusion bodies nor inflammatory lesions can be detected consistently in materials confirmed to be rabies-positive by the IFA technique. Such discrepancies raise questions of the relationship between viral spread, histopathological

changes in the central nervous system (CNS), and clinical signs in rabies infection.

The IFA technique is a rather good method for diagnosis [2], but it has a drawback in that it is difficult to examine histopathological changes in stamps or fresh frozen sections prepared for the technique. To overcome this problem, IFA techniques using enzyme digestion of rabies-infected formalin-fixed tissue sections have been used [11, 20]. However, the sensitivity of viral antigen detection by this method tends to decrease after long preservation [15], so that its sensitivity in such samples is not as high as that in fresh frozen sections.

Recently, immunohistochemical staining techniques have been developed that use specific antisera to rabies virus with peroxidase-antiperoxidase (PAP) [3, 15, 17, 18], and avidin-biotin peroxidase (ABC) [7, 9]. Using these new methods, viral distribution has been examined in natural cases of rabies in humans and animals after death [4, 5].

The mechanisms by which rabies virus disseminates within the CNS are poorly understood, even though rabies virus replication is known to be

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strictly neurotropic. In this aspect, several studies of experimental rabies virus infection have been performed, mainly to clarify the routes of viral spread [6, 8, 13], using ordinary histopathological methods and the IFA technique. Recently, the time-sequential distribution of rabies virus in major regions of the CNS of mice after hindlimb footpad inoculation was attempted using the newly developed ABC method [10].

In the present study, the ABC method was used to examine the distribution of rabies virus antigens in the CNS of mice after inoculation by different routes; intracerebral (ic), intraocular into the posterior chamber (io), intranasal (in), intramuscular (im), and subcutaneous (sc). Since the ic and io inoculations were found to cause fatal infections, analysis of the relationship between viral spread, histopathological lesions in the CNS, and clinical course was carried out mainly on the difference between these two inoculation routes.

MATERIALS AND METHODS

Virus: The CVS strain of rabies virus, which has been used as the standard challenge virus in evaluating the efficacy of rabies vaccine, was prepared as a 20% emulsion of infected mouse brain in saline. The stock virus, with a titer of 10^7 50% tissue culture infectious dose (TCID₅₀)/ml was kindly provided by Dr. N. Hirayama, National Veterinary Assay Laboratory, Tokyo.

Animals and routes of virus inoculation: A total of 63 C3H/He Jms mice at 3 and 4 weeks of age were inoculated with 30 μ l of the stock of rabies virus per mouse, by five different routes: ic, io, in, im, sc. The control animals were inoculated with the same volume of saline. The inoculated animals were observed twice a day.

Antisera: 1) Polyclonal antibody (polyAb): Rabbit hyperimmune serum to rabies virus was kindly provided by Dr. Y. Arai, National Institute of Health, Japan. Its antibody titer, examined by rapid fluorescent focus inhibition test, was 1:20,000.

2) Monoclonal antibodies (mAb): Four types of mAb, of which the specificity was confirmed by immunoprecipitation and Western blotting, were kindly provided by Dr. N. Minamoto, Gifu University. Two mAb, designated NC13-16 and NC12-12, were directed to nucleocapsid protein (NC); these mAb had an IFA titer higher than 1:10,000. The NC13-16 mAb reacted with rabies

virus only, and the NC12-12 mAb reacted with both rabies and rabies-like viruses such as the Lagos and Mokola bat viruses. The other two mAb, G8-7 and G13-13, were directed to glycoprotein (G). The G8-7 mAb reacted with rabies virus strains such as CVS, CVS-chicken-embryo-adapted, RC-HL (vaccine strain), Hep-Flury, Nishigawara, ERA, Komatsukawa (street virus) and 1088 (U.S.A.), and rabies-like virus (Lagos-bat, Mokola). The IFA titer of this mAb was more than 1:100,000 on RC-HL-infected neuroblastoma cells. The G13-13 mAb reacted with rabies virus, with an IFA titer of more than 1:10,000, but not with rabies-like virus. In addition, one mAb directed to matrix protein (M) of the Hep-Flury strain was kindly provided by Dr. A. Kawai, Kyoto University.

Histopathological examinations: When the animals were at the moribund stage or 1, 3, or 5 days after inoculation, they were anesthetized with ether and transcardial perfusion was performed with 10% neutral buffered formalin; the brains, spinal cords, trigeminal nerves and ganglions, and optic nerves were immediately collected. All the specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E).

Immunohistochemical procedures: The ABC-immunoperoxidase technique was used. The tissue sections were attached to glass slides coated with a 1:40 dilution of 0.02% neoprene in toluene, and dried overnight before staining. After deparaffinization and rehydration, all sections were rinsed with 0.01 M phosphate buffered saline (PBS), pH 7.1, and then digested with 0.05% protease for 10 min at room temperature, following which they were blocked for endogenous enzyme activity by incubation in 0.3% hydrogen peroxide in absolute methanol for 30 min. They were then treated with normal goat serum for 30 min to reduce non-specific binding. After the slides were washed with PBS, the first antibodies were applied and the slides were incubated in a moist chamber for 2 hr at 37°C. Control slides were treated with normal mouse ascites or normal rabbit sera instead of the first antibody. After incubation, the slides were washed and then treated with biotinylated sheep anti-mouse or donkey anti-rabbit Ig G (Amersham, UK) diluted 1:200 for 30 min at room temperature. After being washed, they were incubated with horseradish peroxidase-labelled streptavidin biotinylated complex, diluted 1:200 (Amersham, UK), for 15 min. the slides were again washed with PBS, after which they

were treated with 0.5 mg/ml 3-3' diaminobenzidine tetrahydrochloride (Dotite, Wako, Japan) and 0.005% hydrogen peroxide in PBS, counterstained with hematoxylin, and examined under a light microscope.

RESULTS

Clinical outcome : A total of 26 3-week-old C3H/He Jms mice were inoculated with the CVS strain by the ic, io, in, im, and sc routes. As shown in Fig. 1A, all mice inoculated ic and io died by 6 days post inoculation (dpi) and by 9 dpi, respectively. In contrast, all the mice inoculated by the other routes, i.e., sc, im, and in, survived; they were

sacrificed at 23 dpi without having shown any clinical signs, like the control mock-infected mice.

A total of 25 4-week-old mice were inoculated similarly (Fig. 1B). In contrast to the 3-week-old mice, 4 out of 8 mice inoculated ic and 5 out of 6 mice inoculated io survived, and their survival time was 2–4 days longer than that of the 2-week-old mice. All the mice inoculated by the other routes survived. Thus, only the ic and io inoculations resulted in fatal infections in both age groups. The clinical signs were depression, ataxia, and seizure, and the animals died 3 to 6 hr after the appearance of the clinical signs.

Distribution of the viral antigen: Since fatal infection occurred in all 3-week-old mice inoculated ic and io, the subsequent study to examine the time-sequential distribution of virus antigens and histological lesions, was conducted using 3-week-old mice. A total of 22 mice were inoculated with the virus by the ic, io, in, im, and sc routes, and 1, 3, 1, 1 and 1 mice, respectively, were sacrificed at 1 dpi; 2, 3, 1, 1 and 1 mice were sacrificed at 3 dpi; and then 2, 2, 1, 1 and 1 mice were sacrificed at 5 dpi, respectively. All these mice were fixed by the perfusion technique after sacrifice. In addition, 7 mice which were sacrificed at the moribund stage at 6 to 9 dpi in the previous experiment were also included in this study. The organs of these mice were embedded in paraffin for histopathological and immunohistochemical studies. As shown in Table 1, one polyAb and 2 mAb, against NC (NC12–12) and G (G13–13), demonstrated viral antigens in the CNS and in the peripheral nervous system (PNS) of ic- and io- inoculated mice. No staining by the other 2 mAb was observed, even in regions positively stained by the polyAb. The viral antigen stained by

Fig. 1. Survival rate in 3-week-old mice (A) and 4-week-old mice (B) following inoculation by different routes. Numbers in parenthesis indicate the numbers of mice inoculated at 3 weeks old and 4 weeks old, respectively. ●: ic (4, 8), △: io (7, 6), □: sc (5, 0), ▲: im (3, 3), ○: in (2, 4), ★: controls (5, 4).

Table 1. Time-sequential detection of rabies virus antigens in the experimentally infected mice

Inoculation route	No. of mice	Antibody	Reaction by ABC method of virus antigen (dpi)				
			1	3	5	6	7–9
ic	6	poly Ab	—	+	+	+	D ^{a)}
		NC12–12	—	+	+	+	D
		G13–13	—	—	+	+	D
io	14	poly Ab	—	—	+	+	+ ^{b)}
		NC12–12	—	—	+	+	+
		G13–13	—	—	+	+	+
in	3	poly Ab	—	—	—	ND	ND ^{c)}
im	3	poly Ab	—	—	—	ND	ND
sc	3	poly Ab	—	—	—	ND	ND

a) All mice died before sampling.

b) Mice were sacrificed at the moribund stage.

c) Not done.

the polyAb and the mAb to NC was first detected at 3 dpi for the ic route and at 5 dpi for the io route. The mAb to G detected the viral antigen slightly later, at 5 dpi, for the ic route. Mice inoculated by the other routes remained negative for all periods tested by the polyAb. The normal rabbit serum and normal mouse ascites used as controls showed no positive staining.

Histopathological and immunohistochemical examinations: Gross lesions were not found in any of the mice, except for congestion in the brain, which was found in the mice inoculated ic, io at 3 weeks of age that died. Histopathological examination of sections stained with H & E failed to detect any indication of neuronal degeneration, glial reaction, or inflammatory reaction, including perivascular cuffings. No intracytoplasmic inclusions, including Negri bodies were seen. On the other hand, immunoperoxidase staining using one polyAb and 2 mAb, NC 12-12 and G 13-13, detected the presence of viral antigens. The NC protein stained by mAb, NC 12-12, was found in all regions stained positively by polyAb. NC12-12 stained the perikarya of neurons and neuronal processes, glial cells, and ganglion cells. G13-13 stained a few neuronal and glial cells. The staining patterns with these 2 mAb were characterized by fine granules or coarse dots evenly distributed in the cytoplasm. The clinical signs, histopathological lesions and viral antigens in the infected mice are summarized in Table 2. The distribution of the viral antigens is described below.

Distribution of the viral antigens in ic-inoculated mice: The ic-inoculated mice were sacrificed at 1, 3, 5 and 7 dpi. Positive staining for the NC antigen was detected by mAb NC12-12 at 3 dpi in a large number of neurons in the cerebral cortex, thalamus, and spinal cord, in fewer cells in the suprachiasmatic nucleus, medial septal nucleus, and hypothalamus,

and in very few cells in the hippocampus. No positive staining was found in the optic nerve, cerebellum, pontine nuclei, or ganglion cells of the trigeminal nerve. At 5 dpi, the viral antigens were observed more extensively in the above-mentioned positive regions and pontine nuclei but less frequently in the periventricular regions and cerebellum. In addition, larger numbers of viral antigen-positive cells were observed in granular cells of the dentate gyrus and in the pyramidal cells of the hippocampus. The Purkinje's cell and its dendritic processes were also positive at 5 dpi (Fig. 2A). The G antigen detected by mAb, G13-13, was observed to be moderately distributed in the cerebral cortex, thalamus, periventricular hypothalamic nucleus, granular cells of the dentate gyrus, and pyramidal cells of the hippocampus (Fig. 2C). The G antigen was not found in the paraventricular thalamic nucleus, Purkinje's cell, pontine nuclei, or ganglion cells of the trigeminal nerve.

Distribution of the viral antigens in io-inoculated mice: The io-inoculated mice were sacrificed at 1, 3, 5, 7, 8 and 9 dpi. The NC antigen was detected as early as 3 dpi in some ganglion cells and the ocular branch of the trigeminal nerve (Fig. 3A), whereas the optic nerve and tract, cerebrum, cerebellum, and spinal cord were negative at this stage. At 5 dpi, increased amounts of NC antigens were seen in many ganglion cells of the trigeminal nerve, and in neurons of the spinal cord, cerebral cortex, periventricular hypothalamic nucleus, pontine nuclei, and in cerebellar Purkinje's cells and their dendritic processes (Fig. 2B). Moderate staining was observed in the thalamus, and in the hippocampus. No positive staining by G13-13 was observed in the trigeminal nerve ganglions. A few G antigen-positive cells were found in the cerebral cortex, and in the cerebellar Purkinje's cells (Fig. 2D). At 7, 8

Table 2. Summary of clinical signs, histopathological lesions and virus antigens in the experimentally infected mice

Inoculation route	No. of mice	Clinical signs ^{a)}	Mean incubation period (hrs) ^{b)}	Histopathological changes	No. of mice with positive virus antigens				
					poly Ab	NC13-16	NC12-12	G8-7	G13-13
ic	17	+	112(76-119)	—	9/12	0/17	9/17	0/17	0/17
io	21	+	142(74-207)	—	12/12	0/21	12/21	0/21	1/21
in	8	—	S ^{c)}	—	0/3	0/3	0/3	ND	ND
im	9	—	S	—	0/3	ND ^{d)}	ND	ND	ND
sc	8	—	S	—	0/3	ND	ND	ND	ND

a) Clinical signs consisting of depression and ataxia.

b) No. in parenthesis indicate range.

c) Survived for 23 dpi until sacrificed.

d) Not done.

Fig. 2. Distribution of rabies viral antigen in the brain, spinal cord and trigeminal nerve at 5 dpi. Black dots represent viral antigens detected by the ABC-immunoperoxidase method; (A) ic-inoculated mice stained by mAb NC12-12; (B) io-inoculated mice stained by mAb NC12-12; (C) ic-inoculated mice stained by mAb G13-13; (D) io-inoculated mice stained by mAb G13-13.

and 9 dpi, the intensity of staining for both NC and G antigens was markedly increased in neurons, neuronal processes in the cerebral cortex, olfactory bulb, hypothalamus, paraventricular thalamic nucleus, periventricular hypothalamic nucleus, and in Purkinje's cells and their dendritic processes except

the thalamus and granular cells of dentate gyrus of the hippocampus. The gray matter of the spinal cord was also positive. No staining was observed in the optic nerves.

One mouse was sacrificed at the moribund stage at 9 dpi. Severe congestion was found macroscopically in the brain but no inflammatory reaction was found. Both NC and G antigens were observed in all the above-mentioned regions, especially in the paraventricular thalamic nucleus, cerebral cortex, periventricular hypothalamic nucleus, pontine nuclei, and Purkinje's cells, in the gray matter of the spinal cord, in a few cells in the thalamus, and in the granular cells of the hippocampus (Fig. 3B, C, and D). None of the animals survived after day 9.

DISCUSSION

Because of the neurotropic nature of rabies infection [16], knowledge of the distribution of virus antigen in the CNS is essential in understanding its pathogenesis. The ABC-immunoperoxidase method is useful, especially as it can be employed to examine the correlation of viral antigen distribution with histopathological changes [4, 5].

Considering these aspects of rabies infection, the mice were inoculated by the io, im, in, sc and ic routes; the first four of these routes above being those involved in natural infection. Only io and ic inoculations caused fatal infections. We, therefore, carried out a detailed study of the time-sequential distribution of viral antigen following inoculation by these two routes. In io-inoculated mice, the viral antigens spread from a few ganglion cells in the trigeminal nerve at an early stage, 3 dpi, following which they spread to the cerebrum and cerebellum at 5 dpi. Thereafter, the amount of viral antigen increased, except in the thalamus, and the granular cells of the hippocampus in which a decrease was noted in io-inoculated mice but not in ic-inoculated mice. Besides the difference in the thalamus, there were some differences between ic- and io-inoculated mice in the amounts of viral antigens in the paraventricular thalamic nucleus, cerebellum, and pontine nuclei. Except for these differences, the final distribution of viral antigens was similar in both ic- and io-inoculated mice.

The viral antigen appeared in the cerebellum earlier in io-inoculated mice than in ic-inoculated mice. Viral antigens tended to appear in Purkinje's cells but not in granular cells. These results indicate

Fig. 3. Typical patterns of rabies viral antigens detected by mAb NC 12-12 in io-inoculated mice; (A) Ganglion cells of the trigeminal nerve at 3 dpi; (B) neurons and neuronal processes in the cerebral cortex at 9 dpi; (C) pyramidal cells, and in the molecular layer, and granular cells at dentate gyrus of the hippocampus at 9 dpi; (D) the cerebellar Purkinje's cells and their dendritic processes at 9 dpi.

that the deep spinocerebellar tracts and nuclei may serve as a major pathway for the virus; these results agree with those of Gillet *et al.* [8].

Negri bodies which consist of viral nucleoproteins accumulated in the cytoplasm, were not found in this study, even in mice at the moribund stage. The mice may have succumbed before sufficient amounts of viral antigens had accumulated to be detected as Negri bodies by conventional H & E staining. Although the reasons for the absence of Negri bodies are unknown, they might be due to the experimental conditions in this study. Nevertheless,

this result indicates that the absence of Negri bodies does not necessarily deny rabies. Viral antigens were detected in the CNS of moribund mice although inflammatory reactions were absent. Direct effects of virus replication on the functions of CNS regions such as the thalamus, hypothalamus, paraventricular thalamic nucleus, periventricular hypothalamic nucleus, hippocampus, and pontine nuclei may be involved in the onset of clinical signs and fatality.

Some mAb, i.e., the anti-nucleoprotein NC13-16, anti-glycoprotein G8-7, and the anti-matrix mAb

failed to detect viral antigens in regions confirmed to be positive by polyAb or other mAb. This discrepancy may be due to the change in antigenicity of some epitopes during formalin fixation and paraffin embedding treatment.

Usually the hippocampus is used for virus isolation and the detection of viral antigen by the IFA technique for diagnosis. In this study, however, viral antigens were not found in the hippocampus of io-infected mice at the early stage despite the presence of large amounts of viral antigens in the cerebral cortex and cerebellum. Viral antigens were detected in the hippocampus at the late stage. A similar result was reported in mice experimentally infected through a hindlimb footpad [10]. We have encountered false negative diagnoses in natural cases of clinically typical rabies when the conventional IFA technique was used on imprints or smears on glass slides. By using the ABC method on paraffin sections of tissues from these clinical cases, we found only a few antigen-positive cells in the hippocampus while we found a larger number of these cells in the cerebellum (to be published). These results suggest that if only the stamp of the hippocampus is examined for diagnosis, we may overlook viral antigens.

In the experimental infection of rats, it has been demonstrated that rabies virus entered from the peripheral nerves and spread through axonal transport and the cerebrospinal fluid (CSF) pathway [8, 19]. In mice inoculated ic with a high dose of rabies virus, viral antigens were found in the ependymal cells and in neurons adjacent to the central canal of the spinal cord [10]. We did not find positively immunostained ependymal cells in io-inoculated mice, but we did find an increased number of positive cells in the paraventricular thalamic nuclei. This result suggests the possibility of CSF dissemination by io inoculation.

In the io-inoculated mice, viral antigens were detected at early stage in ganglion cells and ocular branch of trigeminal nerve. This finding suggests that the virus spread very early from the posterior chamber of the eyes to the brain, through the ganglion cells in the trigeminal nerve. Viral antigen was not found in the optic nerve in spite of the presence of large amounts of viral antigens in the trigeminal nerve after virus inoculation into the posterior chamber of the eye. Similar findings have been reported for mice experimentally infected with rabies virus via the ocular route; the virus spreading

from the anterior chamber of the eyes to the brain through the trigeminal, oculomotor, and preoptic retinal pathways being shown by the IFA technique [13]. Infection by corneal transplant has been reported in natural cases of rabies [1]. The io infection of mice may provide a suitable model for analyzing the spread of virus by the peripheral route.

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