FULL PAPER *Pathology*

The Effects of Antipyretics on Influenza Virus Encephalitis in Mice and Chicks

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ABSTRACT. To investigate the effect of antipyretics on the murine and poultry models of influenzal encephalitis, we injected large quantities of antipyretics, acetylsalicylic acid (aspirin) and dicrofenac sodium (voltaren). The effect of antipyretic treatment on the murine encephalitis model was unremarkable histologically and immunohistochemically. Whereas in chicks, CNS lesions consisting of perivascular cuffing and gliosis appeared only in those animals treated with the antipyretics and viral antigen was detected mai nly in the nuclei of glial cells or vascular endothelia of voltaren-treated animals. We here demonstrate that antipyretic treatment aggravated the hematogenous spread of the influenza virus to the CNS in chicks, but did not affect the transneural infection in mice.
KEY WORDS: antipyretic, chick, encephalitis, influenza, mouse.

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In humans, the main symptoms of infections by the influenza virus are pyrexia, malaise, and respiratory disorders. Encephalitis/encephalopathy associated with such infections also occurs in children and the elderly. More than 350 cases of influenzal encephalitis/encephalopathy were reported from 1999 to 2001 in Japan [9], but how the virus contributes to the development of brain lesions is unresolved. Antipyretic treatment has been suspected as a enhancing factor for the influenzal encephalitis/encephalopathy, especially in Reye's syndrome, based on epidemiological data [5–7, 18, 20], and the restraint of aspirin treatment for influenza patients resulted in a sharp decline of Reye's syndrome in the U.S.A. [2].

Reye's syndrome has never been documented in animals, and the effects of antipyretic treatments on influenzal encephalitis/encephalopathy have not been fully elucidated in animal experiments [3, 4, 10]. We previously demonstrated that nasal inoculation of a neurovirulent avian influenza virus, strain 24a5b that acquired virulence for chicks and mice through serial passages in air sac and brain of chicks, caused lethal encephalitis in mice and chicks. In the murine model, the virus invades the CNS via vagus nerves after replicating in the respiratory mucosa [14, 15], whereas in the chick model, it spreads hematogenously to the CNS [12, 16]. It would be intriguing to investigate the effect of antipyretics on the murine and poultry models of influenzal encephalitis. In this study, we examined the effects of two kinds of antipyretics, acetylsalicylic acid (aspirin) and diclofenac sodium (voltaren), on these two different animal models of influenzal encephalitis.

MATERIALS AND METHODS

Virus strain: A strain (24a5b) of neurovirulent avian influenza A virus was used in this study. This strain was derived from the original strain A/Whistling swan/Shimane/499/83 (H5N3) after serial passages in the air sac and brain of chicks [16]. The virus was handled exclusively in biosafety level 3 containments in our laboratory.

Antipyretics: Acetylsalicylic acid (ASA), the main component of aspirin and diclofenac sodium (DFS), the main component of voltaren, were used as antipyretics. ASA and DFS were dissolved in PBS and introduced into animals after filter-sterilization.

Animals: Twenty 6-week-old female BALB/cA Jc1 mice (CREA Japan Inc., Tokyo) and thirty 4-week-old male specific pathogen-free chicks were used.

Viral inoculation and medication: Mice were mildly anesthetized with pentobarbital sodium and inoculated with the virus (10^4 50% egg infectious doses: EID_{50}) into both nostrils. Chicks were inoculated into either nostril with 10^2 EID_{50} viruses. Mice and chicks were injected intraperitoneally (IP) with ASA at dosage levels of 700 mg and 500 mg per kg body weight, respectively. DFS was also introduced into mice and chicks at 100 mg per kg body weight by the IP route. The dose levels of the antipyretics were chosen based on the pilot studies in which maximum non-lethal dose levels for mice and chicks were determined.

Experimental design (Fig. 1): Mice were divided into five groups, one inoculated without antipyretic treatment and four inoculated with pre- or post-administration of antipyretics, each comprising four animals. Pre-medication was done 2 days before the inoculation and post-medication, 3 days after the inoculation. Chicks were also divided equally into five groups and treated with the drugs at the same intervals, except that post-medication was done 2 days after the inoculation of virus. The animals were observed for a week after the inoculation and subjected to pathological examinations. Those found to be moribund were necropsied promptly. All animal experiments were conducted in biosafety level 3 containments of our institution according

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to the Guidelines for Animal Experiments established at Hokkaido University.

Histology and immunohistochemistry: The liver, spleen, kidneys, heart, lungs, pancreas, small intestine, trachea, thymus, bursa of Fabricius (chicks) and brain were collected and fixed in neutral phosphate-buffered 20% formalin. The organs were sectioned at a thickness of 4-μm and stained with hematoxylim and eosin (HE) for light microscopic examination. For detection of avian influenza virus antigens in the tissues, all the sections were stained using the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO kit, Nichirei Corp., Tokyo) using rabbit anti-strain 499 [A/Whistling swan/Shimane/499/83 (H5N3)] hyperimmune serum [12, 15] at a 1:2,000 dilution as the primary antibody.

RESULTS

Clinical signs and macroscopic findings: The mice showed transient anorexia and reduced movement after ASA injection, and anorexia, ruffled hair and weight loss after DFS injection. An inoculation of the virus resulted in the clinical signs of anorexia, emaciation, ruffled hair, high respiratory rate and distention of the abdomen from 4 post-inoculation days (PID) in all groups. One mouse in the pre-ASA group died at 5 PID and two mice in the pre-DFS group died at 7 PID. At necropsy, hemorrhagic foci in the lungs and retention of gas in the stomach and intestines were observed in some mice of all groups.

The chicks of all groups showed mild depression and subcutaneous edema in the face and legs at 2 PID. Three chicks and one chick in the post-DFS and pre-DFS groups died at 7 and 3 PID, respectively. Except in the DFS groups in which the clinical signs progressed, all chicks recovered gradually from the signs before 7 PID.

Histological findings: All the mice inoculated with the virus showed mild bronchointerstitial pneumonia characterized by thickening of alveolar walls due to infiltrations of lymphocytes and macrophages, and enlargement of type 2 alveolar epithelial cells. Exfolliated mucosal epithelial cells and neutrophils were found in the alveolar lumina. In the CNS of the inoculated mice, nonsuppurative encephalitis including perivascular cuffing, the vacuolation of neuropils and the necrosis of some nerve cells was observed mainly in the brain stem. Those pulmonary and CNS lesions did not differ in severity among the groups (Fig. 1).

In chicks by contrast, CNS lesions were observed only in the ASA- and DFS-medicated animals, not in those untreated with the antipyretics (Fig. 1). The CNS lesions consisted of perivascular cuffing of lymphocytes, glial nodules with some necrotic cells and vasculitis located mainly in the cerebrum. The glial nodules preferably located around the blood vessels (Fig. 2). Some of the DFS-medicated chicks showed a few to multiple foci of necrosis in the heart, kidneys and pancreas, mild to moderate depletion of lymphocytes in lymphoid organs, mild fatty change of hepatocytes and mild interstitial pneumonia consisting of infiltration of macrophages, lymphocytes and heterophils with pulmonary edema.

Immunohistochemical findings: Immunohistochemical examination of the mice revealed viral antigens in the CNS and rarely in the lungs (Table 1). A few alveolar epithelial cells and macrophages were antigen-positive in the lungs of some mice. In the CNS, the antigens located mainly in the encephalitic foci in the medulla oblongata and less frequently in the pons and diencephalon (Fig. 1). The antigens appeared in the nuclei of nervous and glial cells, but not in vascular endothelial cells.

In DFS-treated chicks, the antigens were detected in the endothelial cells, glial nodules and glial cells around the blood vessels of the CNS (Fig. 2). Antigen-positive cells also existed in the heart, kidneys, pancreas and inflammatory cells of the lungs. No viral antigens were observed in the chicks of other groups (Table 2).

DISCUSSION

Reye's syndrome occurs in childhood after an infection of influenza A or B virus and varicella virus. The characteristics of the disease include cerebral edema and fatty degeneration of viscera, especially in the liver [13]. It has been suspected that the use of antipyretics is related with the development of Reye's syndrome [5–7, 18, 20] and a ban of antipyretic treatment for febrile disease in children has resulted in a drastic decrease in the incidence of the syndrome [2]. However, the exact effects of antipyretics on influenza virus infection remain to be clarified and to the best of our knowledge, the enhancing effect of antipyretics on influenzal encephalitis has not been demonstrated in animal experiments.

The present results of both histological and immunohistochemical examinations on the CNS of mice were consistent with those of our previous reports in which the virus invaded the brain stem after replicating in the respiratory mucosa [14, 15]. Treatment of the mice with the antipyretics did not affect the development of the CNS lesions. On the other hand, in chicks, antipyretic treatments before and after inoculation of the influenza virus definitely enhanced the neuropathogenicity of the virus. The chicks inoculated with the virus without antipyretic treatment developed no lesions and showed no antigen in the CNS. DFS enhanced the neuropathogenicity more than did ASA. However, the present results did not imply the successful reproduction of Reye's syndrome. The dosage levels of the antipyretics in the present experiments were far beyond the therapeutic dosage of the drugs. In addition, histological and immunohistological changes seen in this study were different from the syndrome, in which encephalopathy without viral antigen in the brain and severe fatty degeneration of hepatocytes are hallmarks [5, 13, 19].

Both DFS and ASA are non-steroidal anti-inflammatory drugs (NSAIDs) and non-selective inhibitors of cyclooxygenase (COX)-1 and COX-2. COX-1 is the constitutive form of COX and provides homeostatic functions

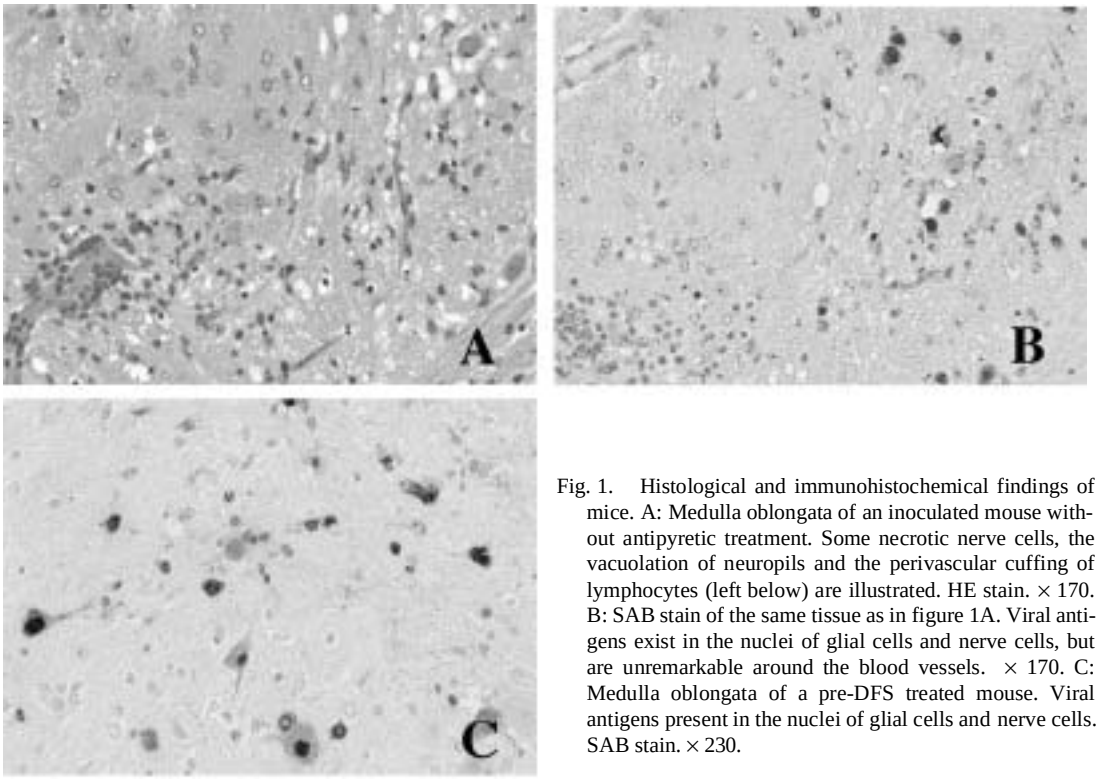

Fig. 1. Histological and immunohistochemical findings of mice. A: Medulla oblongata of an inoculated mouse without antipyretic treatment. Some necrotic nerve cells, the vacuolation of neuropils and the perivascular cuffing of lymphocytes (left below) are illustrated. HE stain. × 170. B: SAB stain of the same tissue as in figure 1A. Viral antigens exist in the nuclei of glial cells and nerve cells, but are unremarkable around the blood vessels. × 170. C: Medulla oblongata of a pre-DFS treated mouse. Viral antigens present in the nuclei of glial cells and nerve cells. SAB stain. × 230.

such as maintaining the gastric mucosa and blood flow. In contrast, COX-2 is an inducible form of COX, known as inflammatory COX [1, 17]. DFS has greater antipyretic or anti-inflammatory functions than other NSAIDs, while ASA inhibits COX-1 [11]. The target molecules of antipyretics, COXs, distribute in the vascular endothelial cells of the CNS under inflammatory conditions [1, 8]. The present experiment suggests the antipyretic treatment does not affect the transneural infection but enhances the hematogeneous spread of the virus to the CNS. Therefore, it may be that the inhibition of COXs, especially COX-2, by antipyretic treatment facilitated the viral infection of vascular endothelial cells of the CNS, resulted in the break down of the blood-brain barrier and invasion of the CNS in chicks.

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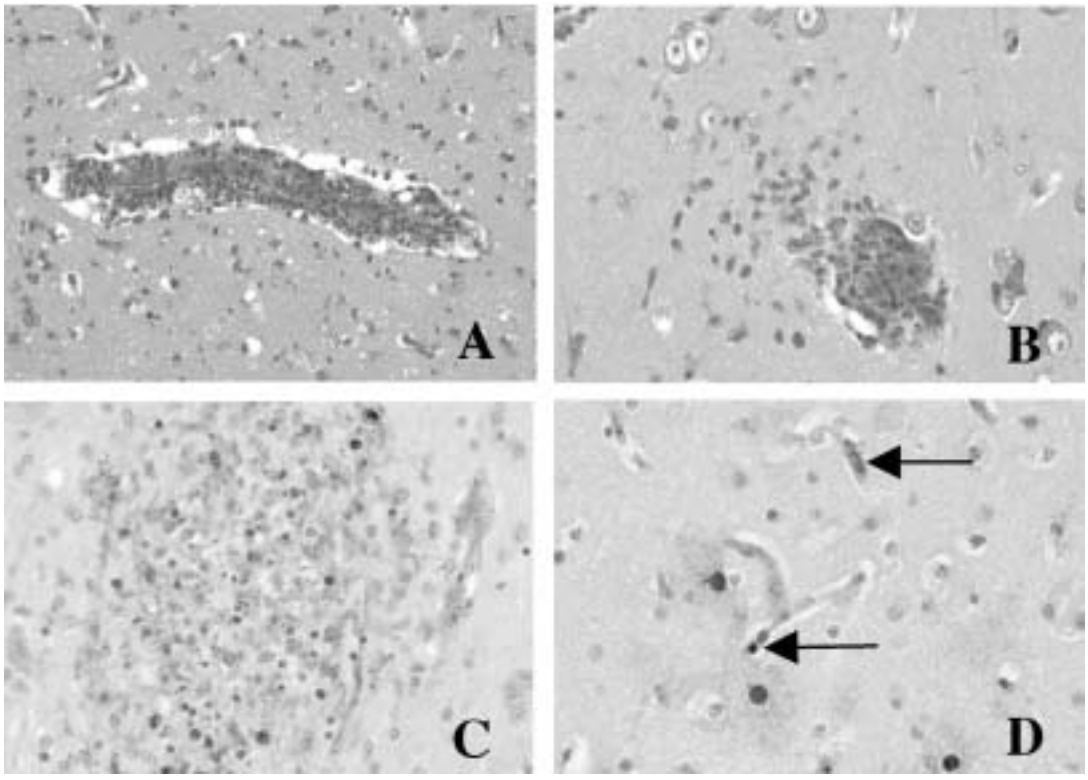

Fig. 2. Histological and immunohistochemical findings of chicks. A: Cerebrum of pre-DFS treated chick. Perivascular cuffing and degenerated glial cells around the blood vessel. HE stain. × 170. B: Cerebrum of post-ASA treated chick. Small glial nodules around the blood vessel with perivascular cuffing. HE stain. × 240. C: Cerebrum of pre-DFS treated chick. Viral antigens in glial nodules. SAB stain. × 250. D: Cerebrum of post-DFS treated chick. Viral antigens in the nuclei of vascular endothelial cells (arrows) and glial cells around the blood vessels. SAB stain × 280.

Table 1. Histological and immunohistochemical findings in the CNS of mice

Groups of mice	Lesions	Viral antigens
Virus alone	4[a)]	4
Pre ASA	4	4
Pre DFS	4	4
Post ASA	3	3
Post DFS	4	4

a) Numbers of mice with lesions and viral antigens per four animals in each group.

Table 2. Histological and immunohistochemical findings in the CNS of chicks

Groups of chicks	Lesions	Viral antigens
Virus alone	0[a)]	0
Pre ASA	3	0
Pre DFS	6	5
Pre ASA	1	0
Post DFS	2	3

a) Numbers of chicks with lesions and viral antigens per six animals in each group.

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