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ORIGINAL RESEARCH

Oseltamivir reduces hippocampal abnormal EEG activities after a virus infection (influenza) in isoflurane-anesthetized rats

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Background: Oseltamivir phosphate (OP, Tamiflu®) is a widely used drug in the treatment of influenza with fever. However, case reports have associated OP intake with sudden abnormal behaviors. In rats infected by the influenza A virus (IAV), the electroencephalogram (EEG) displayed abnormal high-voltage amplitudes with spikes and theta oscillations at a core temperature of 39.9°C to 41°C. Until now, there has been no information describing the effect of OP on intact brain hippocampal activity of IAV-infected animals during hyperthermia.

Objective: The aim of the present study was to examine the effect of OP on abnormal EEG activities in the hippocampus using the rat model of influenza-associated encephalopathy.

Methods: Male Wistar rats aged 3 to 4 weeks were used for the study. Influenza A/WSN/33 strain (1×10^5 plaque forming unit in PBS, 60 µL) was applied intranasally to the rats. To characterize OP effects on the IAV-infected rats, EEG activity was studied more particularly in isoflurane-anesthetized IAV-infected rats during hyperthermia.

Results: We found that the hippocampal EEG of the OP-administered (10 mg/kg) IAV-infected rats showed significant reduction of the high-voltage amplitudes and spikes, but the theta oscillations, which had been observed only at >40°C in OP non-administered rats, appeared at 38°C core temperature. Atropine (30 mg/kg) blocked the theta oscillations.

Conclusion: Our data suggest that OP efficiently reduces the abnormal EEG activities after IAV infection during hyperthermia. However, OP administration may stimulate ACh release in rats at normal core temperature.

Keywords: influenza A virus, oseltamivir, electroencephalogram, slow oscillations, theta oscillations, hippocampus

Introduction

Influenza A virus (IAV) is a common infectious pathogen in humans, which occasionally causes influenza-associated encephalopathy (IAE). IAE is characterized by severe neurological complications, such as convulsive seizures, loss of consciousness, and abnormal behaviors.[1,2]

Oseltamivir phosphate (OP) is a selective neuraminidase inhibitor that prevents influenza virus replication. It is prescribed for seasonal influenza and was the recommended drug for treating the anticipated pandemic of swine influenza (H1N1) in 2009.[3–5] OP works effectively in humans when used within 48 hours following the first appearance of symptoms (fever).[6] However, case reports have associated OP intake with sudden abnormal behaviors.[7–9]

Recent studies have shown that OP and its metabolite, OP carboxylate (OC), cross the blood-brain barrier.[10,11] They have been shown to induce neuronal excitability

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and synchrony in hippocampal slices and slice cultures.[7,12] Moreover, OP increases dopamine levels in the medial prefrontal cortex of rats.[13] Most animal model studies of the action of OP on the brain have been conducted in normal rather than IAV-infected rats or mice, and the animals functioned at the normal core temperature. Until now, there has been no information describing the effect of OP on intact brain hippocampal activity of IAV-infected animals during hyperthermia.

Despite the efficacy of OP in clinics, the putative side effects associated with OP have made the use of this drug controversial. In clinics, IAE patients' electroencephalograms (EEG) are characterized by abnormal high-voltage EEG activity with spikes and theta oscillations during high fever.[14–16] These clinical abnormal EEG activities (high-voltage amplitudes, increased EEG spikes, and theta oscillations) were reproduced in the rat model of IAE under hyperthermia (39.9°C–41°C).[17]

Rats and mice do not normally develop fever after IAV infection.[17,18] In a previous study,[17] abnormal EEG activities were not observed in non-anesthetized IAV-infected rats at the normal core temperature (37°C–38°C). Thus, hyperthermia was a precondition to observe abnormal EEG activities and was induced using a heating system, as previously reported.[17] In the present study, we examined the effect of OP on abnormal EEG activities in the hippocampus of isoflurane-anesthetized rats during hyperthermia, using the rat model of IAE.[17]

Methods

Animals, virus infection and electrophysiology

This study was performed in accordance with the guidelines for animal care and use approved by the animal care committee of The University of Tokushima. Since our goal was to model IAE in children, male Wistar rats aged 3 to 4 weeks were used because rats in this range correspond roughly to the human age range of 2.5 to 3 years.[19]

For viral infection, the rats were anesthetized with ketamine-xylazine (62.6 mg/kg–12.4 mg/kg). Influenza A/WSN/33 strain was stocked (frozen) at –82°C and diluted to obtain the dose (1×10^5 plaque forming unit in PBS, 60 µL), which was then applied intranasally to the rats, as previously reported.[17] The rooms for virus stockage, viral infection, and handling of the infected animals were designed in accordance with the guidelines for animal care and use of The University of Tokushima.

Anesthesia and hyperthermia

The in vivo electrophysiology experiments were carried out under isoflurane anesthesia. EEG activity in isoflurane anesthetized animals is characterized by slow bursting activity followed by a burst suppression period.[17,20]

The rats were first anesthetized by low-dose of ether, then fitted to a stereotaxic frame (model SN-6N, Narishige, Tokyo). The animals were gas-anaesthetized with 1.5–1.7 minimum alveolar concentration (MAC) of isoflurane mixed with 30% O_2 and 70% N_2, which allowed spontaneous respiration. During the EEG recordings, a heating pad system (model 21051-00; Fine Science Tools Inc, Foster City, CA) with a feedback control probe was inserted rectally. The body temperature of rats placed on the heating pad gradually increased from 37°C to 41°C in a period of 20 minutes, as previously reported.[17]

EEG recordings

EEG activity was recorded using enamel-coated tungsten wire electrodes with an uncoated diameter of 120 µm (MT Giken Co, Tokyo, Japan). Craniotomy was performed without damaging the underlying dura using a standard miniature drill equipped with 0.5 mm diameter drill bit. The electrodes were inserted based on the rat brain in stereotaxic coordinates of Paxinos and Watson.[21] To record the hippocampal activity, an electrode was lowered into the CA1-CA3 area (left hemisphere) at 2.8 to 3.0 mm posterior to the bregma, 2.7 to 2.9 mm lateral from midline, and 2.6 to 3.0 mm below the dura. Signals were recorded using a dual microprobe system (WP Manufacturing, Inc, Longmont, CO), and a homemade amplifier (×1000). The baseline was adjusted to zero-level with a slow voltage clamp system with a time constant of 2.2 seconds. The signal was low-pass filtered at 0.5 to 3 kHz, sampled at 1 kHz, and recorded using Axopatch software (Axon Instruments, Palo Alto, CA). To verify the electrode position, the electrode tip was coated with a lipophilic tracer dissolved in dimethylsulfoxide at a concentration of 1 mg/mL to 2.5 mg/mL before insertion into the brain. After removal of the electrodes, the rats were anesthetized with ketamine-xylazine (62.6 mg/kg–12.4 mg/kg) and were transcardially perfused with saline, followed by fixation (4% paraformaldehyde). The brain was removed and immediately put in sucrose and kept in a 4°C room. Sections 800 µm thick were then prepared. Red traces of the dye left by the electrodes were observed under the microscope and photographed.

The electrophysiology EEG data analysis was performed using IGOR Pro 4 (WaveMetrics, Inc, Lake Oswego, OR)

and the fast Fourier transformation (FFT) of EEG activity was computed for 30-second periods.

Slow and distinctive EEG waves were monitored continuously during the experiments to ensure that the rat was well anesthetized and without pain. At the end of experiments, the rats were given a lethal dose of pentobarbital (50 mg/kg).

Characterization of abnormal EEG activities

In order to examine the efficacy of OP on abnormal EEG activities during hyperthermia, we focused particularly on the following observed after IAV infection in the rats during hyperthermia:[17] abnormal high-voltage EEG activity, enhanced low-amplitude fluctuation (LAF) during burst suppressed periods, increased EEG spikes, and theta oscillations. The EEG activity was recorded in the hippocampus of the IAV-infected rats at 8 to 12 hours, 26 to 30 hours, and 50 to 56 hours, corresponding to the periods during which the peak abnormal EEG activities are observed in the rat model of IAE.[17]

The EEG amplitude was measured by quantifying the number of bursts (n = 15 bursts average/point) and the EEG spike was identified as a sharp wave that usually sprouts randomly within the burst and during the burst suppressed periods, as previously described.[17] Theta oscillations amplitude was determined by measuring theta wave amplitude (from the positive to the peak, n = 15 waves average/points). The details of the EEG components and parameters are shown in Figure 1A.

Drug and antagonist treatment

All the rats were infected with IAV. OP was not administered (control) to some of the rats, but was administered to the remaining rats. Tamiflu® capsules (75 mg) were purchased commercially from Chugai Pharmaceutical Co. (Tokyo, Japan), and the OP in the contents of the capsules was dissolved in water. OP mixture with stabilizing additives from the capsules or recrystallized OP was orally administered to the rats and they were monitored (for 1 hour postadministration) in a cage prior to the EEG recording. OP was administered to the rats in a single dose or in two doses per day (Figure 2A).

Atropine (30 mg/kg; Nacarai, Kyoto, Japan), an mAChR agonist, was dissolved in saline (0.9% NaCl) and was given via intraperitoneal (ip) injection.

Statistical analyses

Numerical values were expressed as mean ± SD. P value was obtained by Student's paired t-test (using SigmaPlot 10; Systat Software Inc, San Jose, CA), and $P < 0.05$ was considered statistically significant.

Results

In single dose cases, OP was administered 3 hours after infection (n = 5) and EEG was recorded at 8 hours to 12 hours after infection. In the control rats (IAV-infected rats not given OP), the EEG showed slow oscillations at 38°C core temperature; the corresponding slow component (<1 Hz) is depicted in the FFT plot (Figure 1B, top left). At 40°C core temperature, the EEG showed high-voltage slow EEG activity (or theta-like oscillations in two of the five rats) with increased spikes (arrowheads in the EEG traces) and enhanced amplitudes of the LAF, as shown in the expanded trace (segment 1) (Figure 1B, left bottom). In this study and a previous study,[17] theta-like oscillations in the control rats were observed only during hyperthermia (39.9°C–41°C). In contrast, in the OP-administered rats, the EEG displayed theta-like oscillations at the normal core temperature (38°C) in two out of the five rats (Figure 1B, top right). The FFT plot shows the corresponding theta frequency distributions (Figure 1B, bottom right). At 40°C, the EEG amplitude was reduced and the rhythmic activity of the LAF was disrupted while the amplitude of the LAF was reduced, as shown in the expanded trace (segment 2) shown in Figure 1B. There was a significant difference (n = 3, $P < 0.001$) between the amplitude of LAF values of the OP-administered rats (0.14 ± 0.05 mV) and that of the control rats (0.39 ± 0.06 mV). The quantified abnormal EEG amplitudes (left) and EEG spikes (right) were reduced in the OP-administered rats compared with the control rats (Figure 1B, bottom).

The EEG was then recorded at 26 to 30 hours after infection. Abnormal EEG amplitudes in OP-administered rats were reduced (n = 5, supplementary Figures 1), but the reduction was not significant. However, the amplitude of LAF values (0.26 ± 0.04) was significantly (n = 3, $P < 0.05$) reduced in the OP-administered rats compared to the control rats (0.14 ± 0.04), and the EEG spikes were significantly ($P < 0.001$) reduced in OP-administered rats. This suggests that the efficiency of the single-dose OP administration may be weakened after 26 hours of infection.

When two doses of OP were administered per day, abnormal EEG amplitudes ($P < 0.05$) and EEG spikes ($P < 0.001$) recorded at 26 to 30 hours after infection were more significantly reduced compared with the control rats (n = 3; Figure 2B, top). Those at 50 to 60 hours after infection were also reduced in comparison with the control rats (n = 3, Figure 2B, bottom). This finding suggests that two doses of OP administered per day were more efficient than a single dose in reducing abnormal EEG activities in IAV-infected rats.

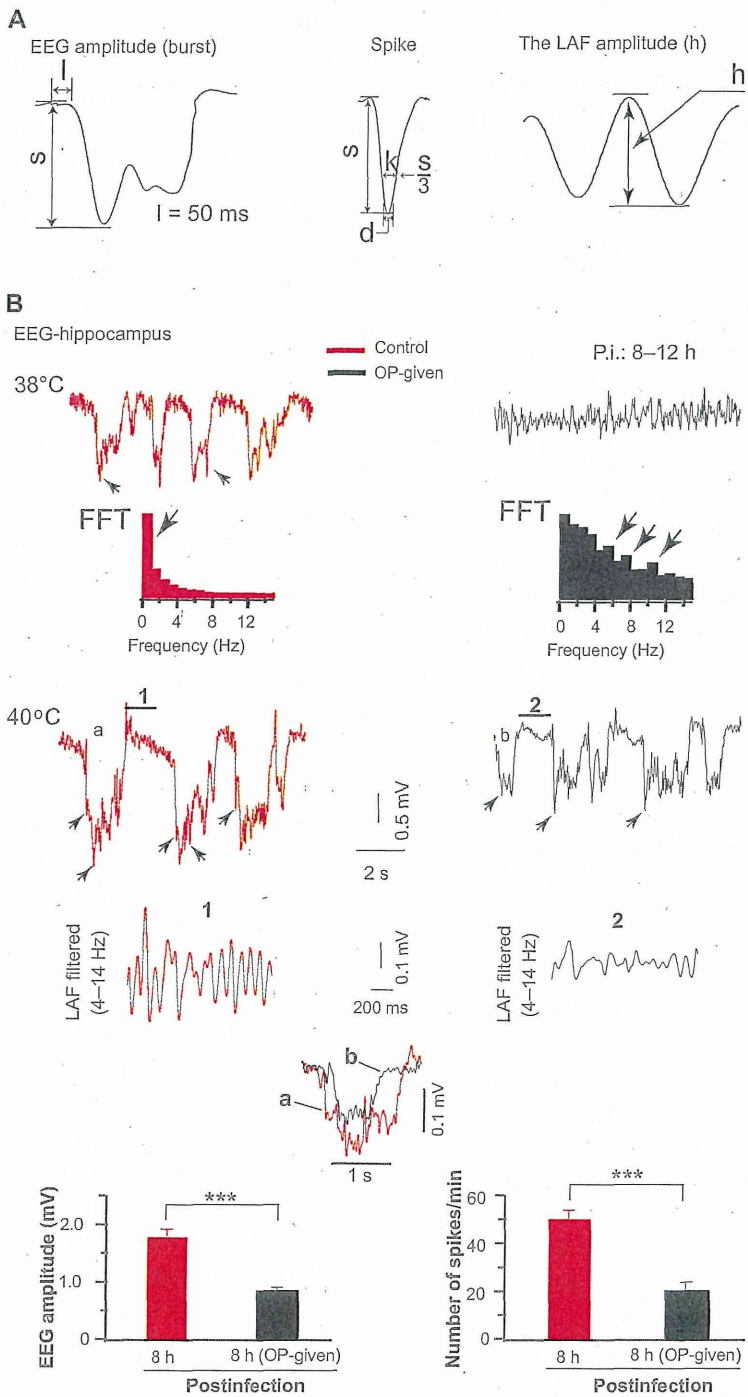

Figure 1 Abnormal EEG activities were reduced in infected rats treated with OP soon after infection. (A) The characterization of abnormal EEG components (S, amplitude in mV, from the base line to depth negative, l = 50 ms); spike (1 ms < d ≤ 15 ms, 1 < k ≤ 35 ms); LAF amplitude (h, amplitude in mV). (B) The recorded abnormal EEG activities at 8 to 12 hours after infection are displayed at 38°C and 40°C body temperatures. Left: At 38°C in the control rats, EEG displayed slow activity and its corresponding dominant slow component (arrow) is depicted in the FFT plot. At 40°C, EEG displayed high-voltage slow EEG activity with enhance LAF amplitude as depicted in the filtered (4–14 Hz) and expanded segment (1, right). In the OP-administered rats, EEG shows theta oscillations (top) at 38°C and the EEG corresponding FFT plot peaked at various theta band frequencies indicated by the arrows (bottom). At 40°C, EEG displayed a low-voltage slow EEG activity. The LAF is shown in the expanded segments (2) filtered at 4–14 Hz. Note a disrupted rhythmic activity and reduce amplitude in the trace (2). Superimposed traces of bursts of the control EEG (a) and of OP-administered EEG (b) displaying a clear reduced amplitude of OP-administered trace. Bottom: Quantification of EEG amplitude (left) and EEG spikes (right) are plotted.

Notes: A significantly decreased EEG amplitudes and number of spikes. Arrowhead in the EEG traces indicates the spike. OP-administered is replaced by OP-given. FFT of EEG activity was computed for periods of 30 seconds. ***$P < 0.001$, ns; n = 3.

Abbreviations: EEG, electroencephalogram; LAF, low-amplitude fluctuation; OP, oseltamivir phosphate; FFT, fast Fourier transformation; ns, nonsignificant.

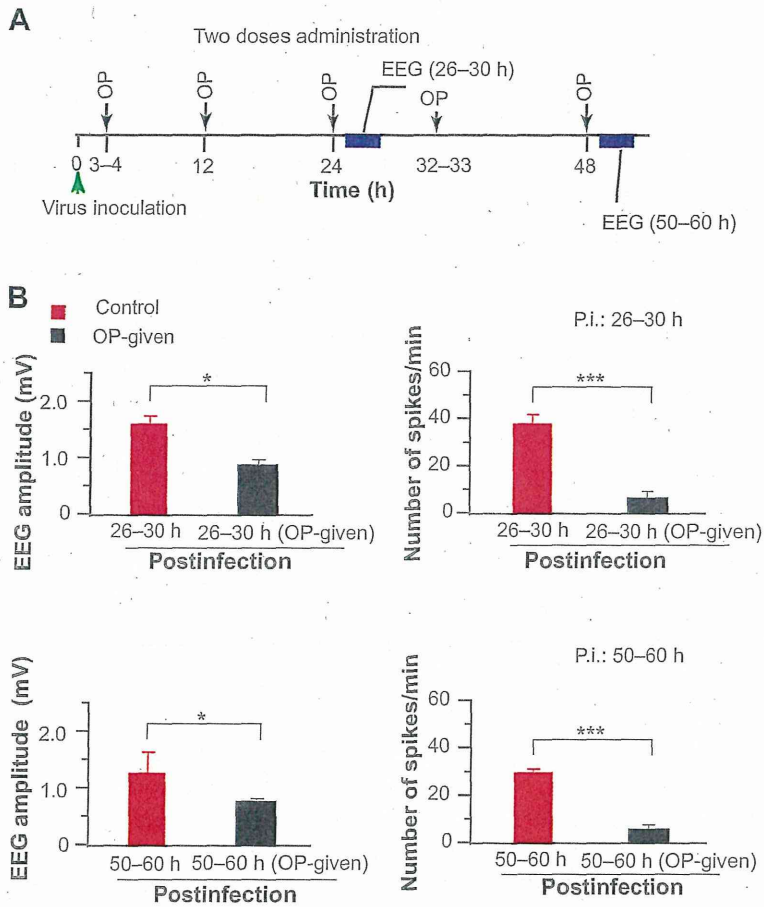

Figure 2 OP significantly reduced abnormal EEG activities. OP applied two doses per day. (A) OP oral administration and EEG recording time course is schematized. The green arrowhead indicates the virus inoculation time, dark arrows show the OP administration time, and the blue rectangle indicates the EEG recording time. (B) Top: EEG activity recorded at 26 to 30 hours after infection.

Notes: The quantification plot shows reduced EEG amplitudes (left) and EEG spikes (right) in the OP-given rats. Bottom: Similar findings and arranged as in top for EEG activity recorded at 50 to 60 hours after infection. OP-given means OP-administered. Data are mean value ± standard deviation of the mean. $^{*}P < 0.05$; $^{***}P < 0.001$; n = 3.

Abbreviations: EEG, electroencephalogram; OP, oseltamivir phosphate.

Two types of theta are known: atropine sensitive (a muscarinic receptor blocker) and atropine resistant.[22,23] We studied the effect of atropine on the OP-induced theta oscillations in IAV-infected rats. We first confirmed that EEG displayed theta oscillations at 38°C. Then, maintaining this temperature, a saline ip solution was injected, and 30 minutes after the saline injection, atropine (30 mg/kg, ip) was administered. As can be seen in Figure 3, atropine blocked the theta oscillation in all the rats examined (n = 3), revealing that the OP-induced theta oscillation is atropine sensitive.

Discussion

The present work is the first study to report the effect of OP on rats infected with IAV under hyperthermia in vivo.

We found that one or two orally administered doses of OP were efficient when given soon (~3 hours) after IAV infection. OP efficiently reduced the abnormal EEG activities (ie, enhanced amplitude of the LAF, high-voltage EEG amplitudes, increased EEG spike) in the IAV-infected rats. However, we also observed theta oscillations at 38°C core temperature in the OP-administered rats.

Although the mechanisms by which OP reduces abnormal EEG activity are not clear, we presume at least that the N-methyl-D-aspartate (NMDA) receptor and the blockage of the virus spread from the host cells could play important roles in reducing the high-voltage amplitudes and EEG spikes, as explained below.

Izumi[7] reported that OP induces neuronal excitability via NMDA receptor activation. Our recent study also found that in uninfected rats the activation of NMDA

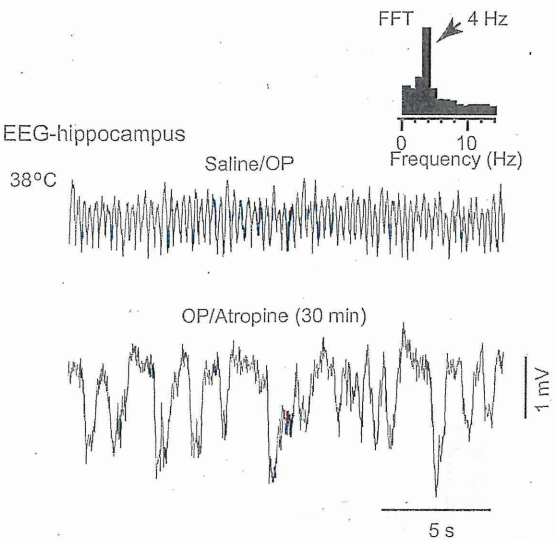

Figure 3 Atropine blocked theta oscillations. The EEG activity was recorded in the hippocampus at 38°C rat body temperature. OP-induced theta oscillations were suppressed by intraperitoneal injection of atropine.
Notes: The FFT plot indicating the theta frequency peak at 4 Hz (top). Atropine blocked theta oscillation in all administered (n = 3) rats. FFT of EEG activity was computed for periods of 30 seconds.
Abbreviations: EEG, electroencephalogram; OP, oseltamivir phosphate; FFT, fast Fourier transformation.

receptors peaked at 2 hours after OP administration and declined at 4 hours after OP administration (data not shown). This suggests the inactivation of the NMDA receptors at 4 hours after OP administration, and this inactivation may prevent calcium influx into the neurons. It is also known that OP is a sialic acid analogue, which inhibits the influenza neuraminidase enzyme and prevents the release and spread of the virus from infected host cells during budding.[4,6] OP treatment (1 hour after infection at daily base) significantly reduces the infection rate in multiple cell types and reduces the progression of the virus in mice.[24] These previous findings suggest that the early administration of OP after IAV infection within a period before the virus proliferates, in addition to the inactivation of the NMDA receptors at a later time (>4 hours) after OP administration, could be possible factors in explaining how OP works to reduce the abnormal EEG activities.

The observation of theta oscillations at 38°C in OP-administered rats is an important finding for confirming the possible action of OP on muscarinic acetylcholine (mACh) receptors. We have observed a similar finding in normal uninfected rats; the OP administered to these rats induced theta oscillations (2 Hz to 6 Hz) at 38°C core temperature (data not shown). Theta oscillations were atropine sensitive and prevented fast hippocampal activity, which resulted in a slow EEG activity (Figure 3). This result is in full agreement with a previously reported in vitro study in which OP was found to activate mACh receptors.[12] The activation of mACh receptors may suggest the stimulation of acetylcholine containing cholinergic neurons, which have been known to play important roles in cortical activation and in regulating states of consciousness.[25,26] ACh is a major excitatory neurotransmitter in the central nervous system. It plays key roles during synaptic transmission, and constitutes a system with other neurotransmitter/modulators to regulate brain states of vigilance.[25]

Because the EEG recording from the hippocampus in nonanesthetized rats was not possible, the effect of anesthesia could be an issue to consider during the interpretation of the data. Isoflurane has a neuroprotective effect on the brain. It reduces excitatory synaptic transmission in the hippocampus,[27–29] and it enhances inhibitory synaptic potentials at concentrations above 0.5 MAC (1%).[29] Isoflurane alters the ACh release in a dose-related manner, and that at 1.5 MAC, it significantly decreases ACh release in the cortex and striatum of rats.[30,31] With such concentrations, EEG activity is generally characterized by slow (bursting) activity followed by burst-suppressed periods.[17,20] In the present and previous studies,[17] 1.5–1.7 MAC isoflurane was used. Thus, it is unlikely that the theta oscillations were isoflurane dependent.

The physiological significance of high-voltage slow and theta oscillations in influenza patients in the clinic is not well documented. However, in the rat model of IAE, we speculated that the alternation between these two oscillations may lead to brain instability, and that this may explain the abnormal behaviors observed in some patients.[17] In the present study, the OP-induced theta oscillations were similar to those observed during hyperthermia in the IAV-infected rats not given OP. Thus, both theta oscillations suggest the stimulation of ACh release. Excessive release of ACh may affect synaptic transmission and oscillation patterns in the brain, which may lead to abnormal behavior in influenza patients. Under such conditions, atropine may play a therapeutic role in stabilizing the brain states from fast (2–6 Hz) to slow (<1 Hz) oscillations.

In the present study, because hyperthermia was a precondition for observing EEG abnormalities in the control rats, the effect of OP on core temperature before and during EEG recording was not investigated. However, recent studies showed that OP induces hypothermia in mice[32] and OP administered in ethanol-injected rats significantly augmented

the hypothermia effect.[7] Hypothermia has also been reported after OP ingestion in clinics.[33]

Conclusion

Our data show that OP, when administered in the early phase of an infection, can efficiently reduce abnormal EEG activities in IAV-infected rats. However, OP administration may stimulate ACh release in rats at the normal core temperature.

Future studies should investigate the biomolecular basis of NMDA and mAChRs expression levels and measure ACh release in OP-administered rats, especially in the cortex and the hippocampus. Such an investigation could improve our understanding of protecting the brain during the early phase of IAV infection against neuronal excitability and neurological complications, and thus prevent abnormal behaviors (which can lead to fatal accidents) in influenza patients with high fever.

Acknowledgments

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Disclosure

The authors report no conflicts of interest, direct or indirect, in this work.

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Supplementary figure

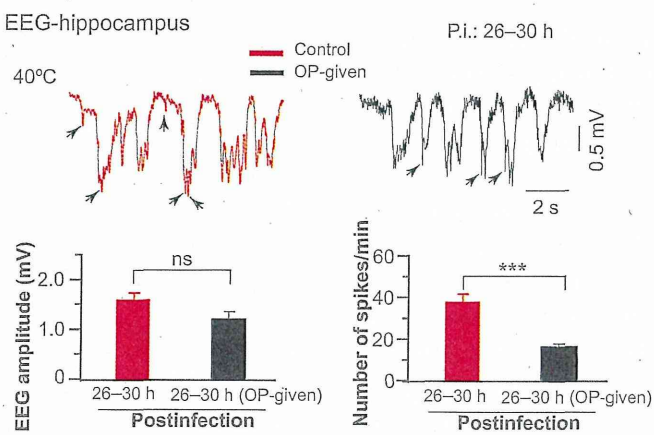

Figure S1 OP significantly reduced EEG spikes but not EEG amplitudes.
Notes: The effect of OP was weakened after 26 to 30 hours postinfection. Note a decrease of EEG amplitude in the OP-administered rats compared with the control rats. However, the difference was not significant. In contrast, a significant decrease in the number of spikes was observed in the OP-administered rats.
Abbreviations: OP, oseltamivir phosphate; EEG, electroencephalogram.

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Original Article

Induction and maintenance of anti-influenza antigen-specific nasal secretory IgA levels and serum IgG levels after influenza infection in adults

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Objectives To determine the induction and changes in anti-influenza virus secretory IgA (s-IgA) levels in nasal washes and serum IgG levels in patients with influenza.

Methods The study recruited 16 patients with influenza aged 35·6 ± 9·6 years in 2007/2008 and 2008/2009 seasons. Nasal washes and serum were obtained throughout the first year. Anti-viral s-IgA levels and neutralization activities in nasal washes, and serum anti-viral IgG levels and hemagglutination inhibition (HI) titers were measured.

Results Anti-viral(H1N1) s-IgA to total IgA ratio and neutralizing antibody titer were low in nasal washes of all patients, whereas serum levels of anti-viral IgG and HI titers varied widely at day 1·4 ± 1·0 postinfection. Both nasal s-IgA and serum IgG levels later increased significantly, reaching peak levels at day 9·6 ± 3·3 postinfection. The induced nasal s-IgA then returned toward the initial levels within 300 days, although the levels at day 143 ± 70 were 3·03-fold of the initial. Individual serum IgG levels also returned toward the initial levels within 300 days, although the mean levels remained high probably because of re-infection in a subgroup of patients. Although influenza A (H3N2) was a minor epidemic subtype in both flu seasons, a significant rise in nasal anti-viral (H3N2) s-IgA levels and a slightly increase in serum IgG levels were noted.

Conclusion Low levels of nasal anti-viral s-IgA and neutralizing antibody were noted compared with a wide range of serum anti-viral IgG and HI titers at the onset of infection. Elevated s-IgA and IgG returned toward the initial levels within 300 days of infection with minor exceptions.

Keywords Influenza virus, mucosal and systemic immunity, secretory nasal IgA, serum IgG.

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Introduction

Influenza, one of the most common infectious respiratory diseases, is associated with considerable morbidity and mortality in infants and aged individuals.[1,2] The first line of defense against respiratory pathogens is mucosal immunity, particularly nasopharyngeal mucosal immunity, which constitutes a major component of the immunological humoral- and cell-mediated responses in the upper and lower airways.[3] All currently available influenza vaccines, with the exception of the cold-adapted live flu vaccine, are administered intramuscularly or subcutaneously, induce a predominantly IgG-mediated protection in the systemic immune compartment, but result in inadequate induction of anti-viral secretory IgA (s-IgA) antibodies with a wide cross-protection activity at the airway mucosa.[4,5]

It has been reported in many of mucosal vaccination experiments in animals, such as nasal influenza A virus (IAV) vaccination, that anti-viral mucosal s-IgA in nasal washes and/or bronchoalveolar fluid is the primary defense compound against IAV infection and that anti-viral s-IgA levels in the airway determine infection sensitivity of animals.[6–9] However, there is little clinical data on s-IgA in nasal washes and bronchoalveolar fluid, and several

questions remain unanswered: (i) What is the time course of mucosal s-IgA induction in nasal washes of patients after IAV infection? and Are there differences in anti-IAV s-IgA levels among infants, children, adults, and the elderly populations? (ii) How long do the induced anti-viral s-IgA levels in nasal washes and serum IgG levels persist after IAV infection? (iii) What are the levels of anti-IAV-specific s-IgA in nasal washes of human that can provide adequate protection against IAV infection? As a first step in answering these questions, we reported recently a new method for sampling nasal washes (nasopharyngeal aspiration) suitable for such studies.[10]

The aim of this study was to define the time course of anti-IAV-specific s-IgA induction as well as serum IgG induction over a period of approximately 1 year after seasonal IAV infection in adults. The results indicated that all patients tested with influenza had low levels of anti-IAV s-IgA and neutralizing antibody in nasal washes at the initial onset of illness, despite the wide-range distribution of anti-IAV IgG levels and hemagglutination inhibition (HI) titers in serum. The induced both s-IgA in nasal washes and IgG in serum returned toward the initial levels during the 300-day postinfection period, with minor exceptions.

Patient population

A retrospective study of 16 adult patients (six men and 10 women; 24–60-year old; mean age: 35·6 ± 9·6 years) was conducted. They suffered from IAV infection during the 2007/2008 and 2008/2009 flu seasons. They were diagnosed with influenza by clinical signs and symptoms and the rapid diagnosis Espline Influenza A&B-N kit (Fujirebio Inc., Tokyo, Japan) on admission. Analysis of the most common features of influenza in this group showed fever (defined as body temperature ≥38°C) in 56% of the patients, pharyngitis in 81%, cough in 75%, nasal discharge in 100%, headache in 69%, and general malaise in 50% of the patients. Informed consent was obtained for enrolment in the study and for the use of stored nasopharyngeal aspirates and sera for quantitative analysis of antibodies. The study was approved by the Committee for Medical Ethics of Tokushima University Hospital. Eleven patients were infected in the 2007/2008 flu season and five were in the 2008/2009 flu season. In both flu seasons, IAV H1N1 subtype was prevalent in Japan.[11,12] Treatment was initiated within 48 h of the onset of fever and included 5-day course of oseltamivir in 12 patients and zanamivir in four.

Sample collection

The nasal washes were collected from patients by using the nasal spray and aspiration method described recently.[10] Briefly, the nasal washes were collected by aspiration from both nostrils over 1-min period from each side through a silicon tube (1·7 mm in diameter, MD-33105; Akita-Sumitomo Bake Co., Akita, Japan) and then trapped in a centrifuge tube connected to an evacuator (EP-1500; Bluecross Co., Saitama, Japan). Finally, the silicon tube interior was rinsed with 1·0 ml of saline, and the collected lavage fluid was immediately cooled on ice, sonicated for 1 min, and centrifuged at 700 ***g*** for 10 min at 4°C. The supernatants were then stored at −30°C until use. Serum samples were simultaneously collected from each patient and stored at −30°C until use.

Nasal washes and serum were again collected from the same patients 2–4 times within the first year after the original infection. The obtained samples were divided into four time periods (Table 1): group 1 (samples collected within 3 days after the onset of illness, mean 1·4 ± 1·0 days, ±SD), group 2 (samples collected between 4 and 21 days after the onset of illness, mean, 9·6 ± 3·3 days), group 3 (samples collected at between 22 and 300 days after the onset of illness, mean, 143 ± 70 days), and group 4 (samples collected more than 300 days after the onset of illness, mean, 368 ± 25 days).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of total IgA and anti-IAV-specific s-IgA in nasal washes and those of anti-IAV-specific IgG in serum were measured by ELISA as described previously[7,13] with minor modifications. The concentrations of total IgA were measured using a human IgA kit (Bethyl Laboratories, Montgomery, TX, USA) according to the instructions supplied by the manufacturer. For measurement of anti-IAV-specific antibody, the prevalent IAV strains were selected as coating ELISA antigens. In the 2007/2008 flu season, IAV/Solomon Islands/3/2006(H1N1)-like subtype was identified in 88% of Japanese cases,[11] while in the 2008/2009 flu season, IAV/Brisbane/59/2007(H1N1)-like subtype was identified in 64% of Japanese cases.[12] Although the IAV/H3N2 subtype was a minor subtype in both flu seasons, most epidemic strains were IAV/Hiroshima/52/2005(H3N2)-like subtype in the 2007/2008 season and IAV/Uruguay/716/2007(H3N2)-like subtype in the 2008/2009 season.[11,12] Therefore, for analysis of anti-IAV-specific s-IgA and IgG in patients of the 2007/2008 flu season, A/Solomon Islands/3/2006(H1N1) and A/Hiroshima/52/2005(H3N2) split-product HA vaccine antigens were used for coating in ELISA, while A/Brisbane/59/2007(H1N1) and A/Uruguay/716/2007(H3N2) split-product HA vaccine antigens were used for ELISA analysis of samples in the 2008/2009 flu season.

For quantification of anti-IAV-specific s-IgA in nasal washes, 96-MicroWell Plates (Nalge Nunc International, Tokyo, Japan) were coated with the IAV vaccine antigens (0·1 μg/well) described previously, incubated overnight in

Table 1. Changes in s-IgA levels against viral antigens and neutralization activity in nasal washes and in serum levels of IgG antibodies and HI titers of IAV-infected patients during follow-up

Days after onset of illness	Within 3 days (<3 days)	4–21 days (<21 days)	22–300 days (<300 days)	>300 days
Nasal lavage fluid (U/μg total IgA × 100)				
s-IgA against A/H1N1	5·9 ± 2·9	31·4 ± 32·0*	17·9 ± 10·4*	8·6 ± 4·3
s-IgA against A/H3N2	4·3 ± 1·3	20·9 ± 19·0*	21·0 ± 11·3*	10·4 ± 4·9
Neutralization activity against A/H1N1 (titer)	6·0 ± 10·8	40·3 ± 47·6**	27·9 ± 4·1	6·0 ± 2·8
No. of samples	8	16	9	4
Sampling after onset of illness (days)	1·4 ± 1·0	9·6 ± 3·3	143 ± 70	368 ± 25
Serum (U/ml)				
IgG against A/H1N1	420 ± 324	858 ± 366*	837 ± 375**	1019 ± 628
IgG against A/H3N2	493 ± 456	847 ± 412	794 ± 353	657 ± 211
HI titer against A/H1N1	44 ± 32	89 ± 155	34 ± 19	47 ± 31
HI titer against A/H3N2	41 ± 49	37 ± 35	40 ± 16	53 ± 23
No. of samples	8	16	10	3
Sampling after onset of illness (days)	1·4 ± 1·0	9·6 ± 3·3	146 ± 66	370 ± 31

Values are mean ± SD.
*P < 0·01, **P < 0·05 versus the values of within 3 days.

phosphate-buffered saline (PBS) at 4°C, and then blocked with 1% BSA in 50 mm Tris–HCl (pH 8·0) containing 0·14 m NaCl and 0·05% Tween-20 (TTBS) for 1 h at room temperature. The nasal wash diluted with TTBS was added to each well and incubated for 2 h at room temperature. The plates were washed five times with TTBS, incubated with goat anti-human IgA conjugated with horseradish peroxidase (Bethyl Laboratories) for 1 h at room temperature, and incubated again with a TMB Microwell Peroxidase Substrate System (KPL Inc., Gaithersburg, MD, USA) according to the instructions provided by the manufacturer. The produced chromogen was measured at an absorbance of 450 nm using a SpectraMax Plus^{384} autoreader (Molecular Devices Corp., Sunnyvale, CA, USA). Multiple samples taken from each patient were simultaneously evaluated in the same plate. Fluctuations in absorbance among plates were adjusted by control analyte. As the affinity-purified human anti-IAV-specific s-IgA standard for each IAV subtype is not commercially available, the anti-IAV-specific IgA concentrations in the nasal washes were determined from the standard regression curves with human IgA of known concentration in a human IgA quantitation kit (Bethyl Laboratories). The relative value of anti-IAV-specific s-IgA was expressed as units (U); 1 U of anti-IAV-specific s-IgA was determined from the regression curve as the point corresponding to 1 μg of human IgA detected in the assay system, as described previously.[13]

As the concentration of nasal wash samples varies widely between individuals depending on the aspiration efficiency, history of nasal diseases, and patient age, the concentration of anti-IAV-specific s-IgA (U/ml) was normalized by the total IgA (μg/ml) concentration in the same sample of nasal washes. The values of normalized anti-IAV-specific s-IgA were highly reproducible in comparison with the values of anti-IAV-specific s-IgA normalized by the amount of protein in nasal washes, particularly in the chronic nasal disease patients. Therefore, the serial changes in the nasal concentration of anti-IAV-specific s-IgA throughout the study period were expressed relative to that of anti-viral IAV-specific s-IgA to total IgA = [anti-IAV-specific s-IgA levels (U/ml)/total IgA (μg/ml) × 100].

The ELISA procedure was also used to determine serum anti-IAV-specific IgG levels. As there was no significant variability in serum protein concentrations, the row values of anti-IAV-specific IgG concentrations (U/ml) were used in the time course analysis.

Assay of hemagglutination inhibition and neutralizing activity

To measure hemagglutination inhibition (HI) activities, serum samples were treated overnight with receptor-destroying enzyme (Denka Seiken Co., Tokyo, Japan) at 37°C to eliminate non-specific HI factors, and the assay was conducted according to the protocol for HI testing established by the World Health Organization.[8] For neutralizing activities of nasal washes and serum, IAV/Solomon Islands/3/2006(H1N1) at 200 PFU and IAV/Brisbane/59/2007(H1N1) at 200 PFU, which were provided by the National Institute of Infectious Diseases of Japan, were incubated with 100 μl of diluted samples at room temperature for 30 min. Then the virus titers in the serially

diluted neutralization mixtures were measured by the plaque assay, as described previously.[7,14]

Statistical analysis

Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test or Welch's *t*-test. A *P*-value <0·05 was considered statistically significant.

Results

Low levels of anti-IAV(H1N1)-specific s-IgA and neutralizing antibody in nasal washes and wide variation in serum levels of anti-IAV(H1N1)-specific IgG and HI titers at the onset of illness

In the 2007/2008 and 2008/2009 winter flu seasons, the IAV H1N1 subtype was the most prevalent in Japan. We analyzed the ratio of anti-IAV(H1N1)-specific s-IgA to total IgA and neutralizing antibody in nasal washes as a major local defense antibody at the site of virus entry, serum HI titer, and serum levels of anti-IAV(H1N1)-specific IgG, a major systemic defense antibody in adult IAV-infected patients at 1·4 ± 1·0 days (onset of illness). The ratio of anti-IAV(H1N1)-specific s-IgA in nasal washes was low in all samples tested (5·9 ± 2·9, anti-IAV(H1N1) s-IgA U/μg of total IgA × 100), with an upper limit of 12·0. In addition, the titer levels of neutralizing antibody in nasal washes were also low at 6·0 ± 10·8. However, serum levels of anti-IAV(H1N1)-specific IgG and HI titers varied from very low to high (Table 1 and Figures 1 and 2); in fact, the HI titers of five of eight IAV-infected patients were relatively high (≥40) at the onset of illness.

Kinetics of induction of nasal anti-IAV(H1N1)-specific s-IgA and serum IgG after IAV infection

Influenza A virus infection resulted in significant induction of nasal anti-IAV(H1N1)-specific s-IgA and neutralizing antibody titers, and serum anti-IAV-specific IgG and HI titers after infection, reaching peak levels between days 4 and 21 (Table 1 and Figures 1 and 2). These results are almost identical to those reported in the kinetics studies on antibody production in mice after induction of nasal IAV infection[15] and also in children vaccinated with live attenuated IAV.[16] Serum HI titers in all except three patients increased after infection, particularly in patients with HI titers ≥80 at the onset of illness. However, a mild or no increase in HI titer after infection was observed in patients with HI titers of <80 at the onset of illness (Figure 2B). Subsequently, the increased nasal s-IgA level in each patient returned toward the respective initial level, although the mean levels of nasal s-IgA and neutralizing antibody titers were still 3·03- and 4·65-fold higher than the initial values, respectively, within the 300-day after the onset of illness

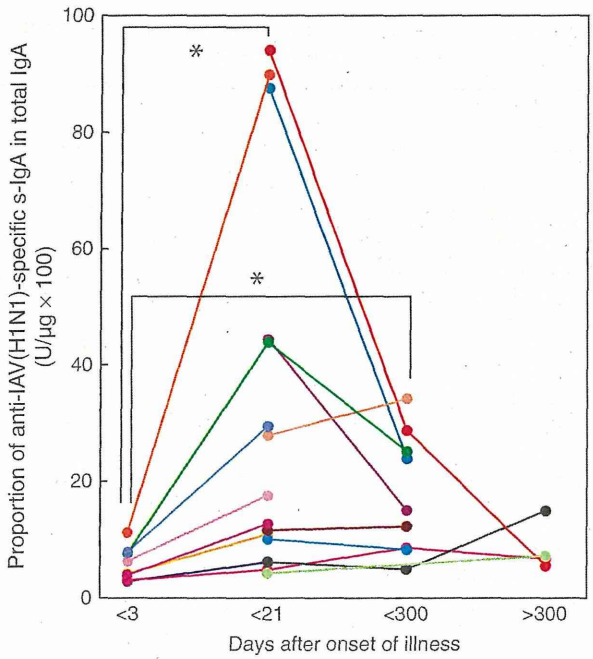

Figure 1. Kinetics of induction of anti-IAV(H1N1)-specific s-IgA in nasal washes after IAV infection examined over a period of about 1 year. Data and lines represent the serial changes in anti-IAV(H1N1)-specific s-IgA/total IgA ratio (U/μg × 100) of individual patients with different color symbols. *P < 0·01.

(Table 1). At 368 ± 25 days postinfection, nasal s-IgA levels of three of four patients were within the initial levels, but the s-IgA of one patient had increased (Figure 1).

The high levels of serum anti-IAV(H1N1)-specific IgG and HI titer in each patient (who showed peak levels during 4 and 21 days after infection) returned toward the initial levels during the follow-up period (<300 days postinfection) (Figures 2A and 2B). However, serum HI titers of five of 10 patients and those of two of three patients were ≥40 between 22 and 300 days and after 300 days postinfection, respectively. These high values from the patients increased the mean HI titers of anti-IAV(H1N1) throughout the follow-up period (Figure 2B and Table 1).

Serial changes in nasal anti-IAV(H3N2) s-IgA and serum IgG after infection

Although IAV(H3N2) was a minor epidemic subtype in both the 2007/2008 and 2008/2009 winter flu seasons in Japan, the kinetics of anti-IAV(H3N2)-specific s-IgA induction in nasal washes and serum IgG induction were similar to those of nasal anti-IAV(H1N1)-specific s-IgA and serum IgG, although the changes in the levels were relatively mild in anti-IAV(H3N2) s-IgA/IgG than those in anti-IAV(H1N1) s-IgA/IgG (Figures 3 and 4). While changes in

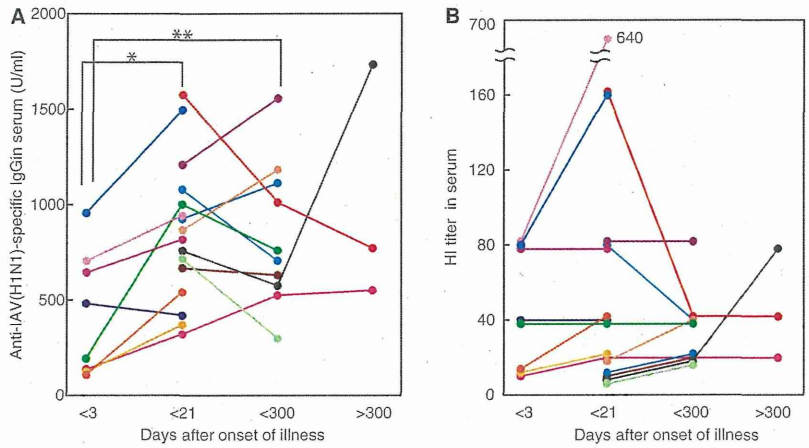

Figure 2. Kinetics of induction of anti-IAV(H1N1)-specific IgG (A) and HI titers (B) in serum after IAV infection examined over a period of about 1 year. Data and lines represent the serial changes in anti-HA(H1N1)-specific IgG (U/ml) of individual patients with different color symbols. *$P < 0{\cdot}01$, **$P < 0{\cdot}05$.

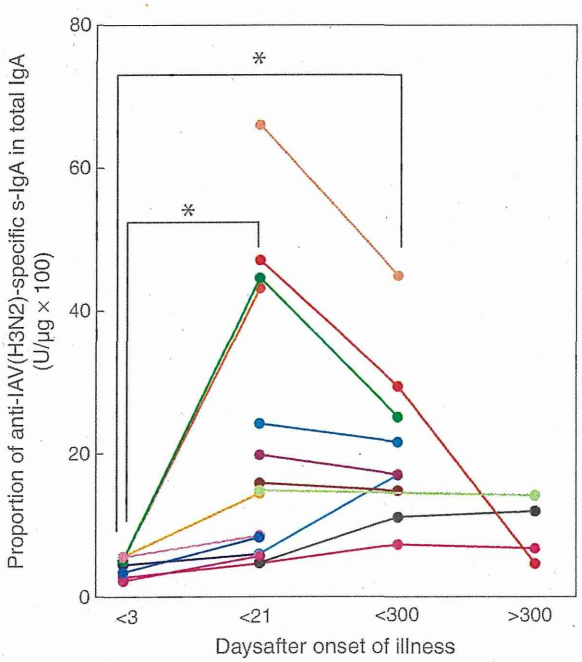

Figure 3. Kinetics of induction of anti-IAV(H3N2)-specific s-IgA in nasal washes after IAV infection examined over a period of about 1 year. Data and lines represent the serial changes in anti-HA(H3N2)-specific s-IgA/total IgA ratio (U/μg × 100) of individual patients with different color symbols. *$P < 0{\cdot}01$.

serum anti-IAV(H3N2) IgG levels were observed during the follow-up period, almost no changes in the levels of HI titer were observed, with several minor exceptions (Figure 4B). Thus, the mean serum HI titers against IAV(H3N2) did not change during the period (Table 1).

Discussion

Our study clearly showed that anti-viral s-IgA levels and neutralizing antibody titers in the nasal washes were low at the onset of infection, irrespective of the levels of anti-viral IgG and HI titers in the serum. In particular, serum HI titers in over half of IAV-infected patients were ≥40 at the time of infection, a critical value for evaluation of the efficacy of influenza vaccine[17] and a value reported to be associated with protection from influenza illness in up to 50% of patients in human challenge studies.[18] These results suggest that nasal s-IgA levels associate with the infectivity of IAV. In the present study, anti-viral s-IgA levels in nasal washes were expressed relative to the total IgA [anti-IAV-specific s-IgA (U/ml)/total s-IgA (μg/ml) × 100], because the concentration of nasal washes varied among the patients. The mean ratio at the onset of illness was $5{\cdot}9 \pm 2{\cdot}9$ (U/μg × 100), and the maximal value in the group was 12·0. These anti-viral s-IgA levels were almost consistent to those reported previously in 10 pediatric IAV-infected patients within 48 h of the onset of fever.[13]

It has been reported that mucosal s-IgA is primarily involved in cross-protection of the mucosal surface against variant IAV infection, and the mechanism of broad-spectrum cross-protection could be explained by the wide-range cross-reactivity of s-IgA.[19–23] We recently found cross-neutralization activity for nasal fluids of minipigs after intranasal vaccination of IAV antigen.[8] In the present study, we found patients with increased levels of s-IgA induction against both H1N1 and H3N2 subtypes, although IAV H1N1 was the predominant subtype in the 2007/2008 and 2008/2009 flu seasons in Japan. The results could probably be explained by the following (although we did not identify

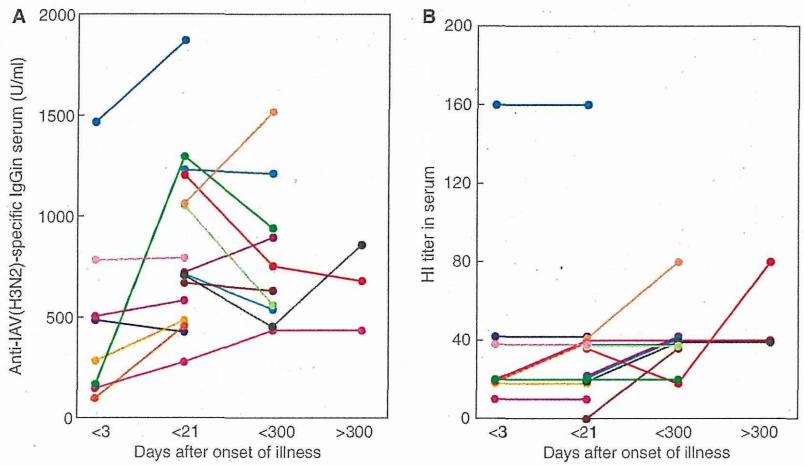

Figure 4. Kinetics of induction of anti-IAV(H3N2)-specific IgG (A) and HI titers (B) in serum after IAV infection examined over a period of about 1 year. Data and lines represent the serial changes in serum anti-HA(H3N2)-specific IgG (U/ml) levels of individual patients with different color symbols.

the subtype of the infecting IAV strain in the clinical samples): (i) The induced s-IgA in nasal washes has cross-reactivity between H1N1 subtype and H3N2 subtype in a manner similar to the reported cross-reactivity in nasal fluids of animals after intranasal vaccination.[8] (ii) Some of the epitopes of H1N1 subtype resemble those of H3N2 subtype and induce heterosubtype immunity by stimulation of pre-existing immunological s-IgA memory.[24] (iii) As we used a split-inactivated viral preparation as an antigen for ELISA assay, it is possible that the induced s-IgA is the antibody against common viral proteins other than hemagglutinin and neuraminidase.

To our knowledge, there are only a limited number of reports on the serial changes in nasal immune responses in patients with influenza. Nasal vaccination has been reported in clinical studies using virosome-based influenza vaccine,[20] a Proteosome™-trivalent inactivated influenza vaccine[21] (ID Biomedical Corporation of Québec, laval, QC, Canada) and a live attenuated IAV vaccine.[16] Furthermore, only little information is available on the serial changes in s-IgA concentration of patients after IAV infection.[25] To determine the kinetics of mucosal and systemic antibody induction in response to the IAV(H1N1) subtype infection in 2007/2008 and 2008/2009 flu seasons, we analyzed the levels of anti-IAV(H1N1)-specific s-IgA in nasal washes and those of IgG in serum of patients over a period of about 1 year.

The induced levels of nasal s-IgA and serum IgG antibodies at day 9·6 ± 3·3 after infection were significantly higher than those at day 1·4 ± 1·0 postinfection. It is possible to explain that the levels at day 1·4 ± 1·0 represent pre-infection levels, because nasal s-IgA level is known to increase rapidly 4 days after nasal virosome-based influenza vaccination[20] and 1 week after treatment of patients with IAV(H1N1) cold-adapted reassortant virus.[16] Further follow-up analysis of individual patients showed a decrease in nasal anti-IAV-specific s-IgA levels before 300 postinfection days, and the average levels of anti-IAV-specific s-IgA and neutralizing activity at day 143 ± 70 were about 57% and 69% of the peak level, respectively. The levels subsequently returned to the pre-infection levels after about 1 year (368 ± 25 day). The results are in general agreement with those of Shvartsman and Grigorieva[25] who reported persistently high titers of antibodies against IAV in nasal secretions for 4–8 months in mild cases of infection and for more than 8 months in severe cases complicated with pneumonia. Induced serum anti-IAV(H1N1)-specific IgG levels were also decreased in a large number of patients, although slowly, during 1-year follow-up period, but the mean values in Table 1 were 0·98- and 1·2-fold of the peak levels recorded at days 146 ± 66 and 370 ± 25, respectively. These findings were the reflection of data of a small group of patients who showed an increase in serum IgG levels, HI titers, and nasal s-IgA levels during the follow-up period, probably due to IAV re-infection. Although we tried to detect the viral re-infection by the rapid diagnosis Influenza A&B-N kit after the initial series of experiments for about 1 year, viral antigen was not detected probably because of long-term storage of nasal washes after sampling. The results of the present study on the kinetics of antibody induction by natural IAV infection (Table 1) are roughly consistent with the data of live influenza vaccination.[26,27] In these studies, both nasal s-IgA and serum IgG levels reached maximum values at 1 month, then gradually decreased to pre-immunization levels in 6 months.

We have recently reported attenuation of inducible respiratory immunoresponses by oseltamivir treatment in mice

infected with IAV[9] and non-significant increases in anti-IAV-specific s-IgA in nasal washes of pediatric IAV-infected patients treated with oseltamivir for 5 days.[13] These results are considered to be because of the suppression of viral replication and viral antigen production by the anti-viral agent. However, adult patients in the present study (age, 35·6 ± 9·6 years), who were treated with oseltamivir or zanamivir for 5 days within 48 h after onset of illness, showed significant increase in s-IgA in nasal washes and IgG in serum at day 9·6 ± 3·3. These findings suggest that despite the antiviral treatment and probably the low production of viral antigen doses in mucosal loci, nasal s-IgA antibodies increase after stimulation of pre-existing immunological s-IgA memory in adult patients, but not in naïve children aged 5·9 ± 3·3 years reported previously.[13]

In summary, our results showed low nasal levels of anti-IAV-specific s-IgA and neutralizing antibody in all tested patients who were infected with IAV at the onset of illness, although highly variable serum levels of anti-viral IgG and HI titers were observed. The induced nasal s-IgA levels and neutralizing antibody titers returned toward the initial levels during the 300-day postinfection period, and the mean levels of anti-IAV s-IgA and neutralizing antibody titer were 3·03- and 4·65-fold of the initial levels, respectively, at postinfection day 143 ± 70. Induced serum IgG levels in the majority of patients slowly returned toward the initial levels during the 300-day postinfection period, although some patients were found to have high anti-viral IgG levels and HI titers in serum probably due to IAV re-infection. Taken together, the findings presented in this study provide important information on s-IgA levels with regard to the sensitivity to IAV infection as well as data that could be potentially useful for evaluation of intranasal IAV vaccination.

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Conflict of interest

The authors declare no conflicts of interest.

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Original article

Epidemiology of acute encephalopathy in Japan, with emphasis on the association of viruses and syndromes

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Abstract

A research committee supported by the Japanese government conducted a nationwide survey on the epidemiology of acute encephalopathy in Japan using a questionnaire. A total of 983 cases reportedly had acute encephalopathy during the past 3 years, 2007–2010. Among the pathogens of the preceding infection, influenza virus was the most common, followed by human herpesvirus-6 (HHV-6) and rotavirus. Among syndromes of acute encephalopathy, acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) was the most frequent, followed by clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS), acute necrotizing encephalopathy (ANE) and hemorrhagic shock and encephalopathy syndrome (HSES). Influenza virus was strongly associated with ANE and MERS, HHV-6 with AESD, and rotavirus with MERS. Mortality was high in ANE and HSES, but was low in AESD, MERS and HHV-6-associated encephalopathy. Neurologic sequelae were common in AESD and ANE, but were absent in MERS.

Keywords: Acute encephalopathy; Epidemiology; Acute necrotizing encephalopathy; Acute encephalopathy with biphasic seizures and late reduced diffusion; Clinically mild encephalitis/encephalopathy with a reversible splenial lesion

1. Introduction

Acute encephalopathy is a severe complication of common infections of childhood, such as influenza, exanthem subitum and acute viral gastroenteritis. It usually affects children who have previously been healthy, and often causes death or severe neurological handicaps. There are two classifications of acute encephalopathy [1]. One is based on the pathogen of the preceding infection, such as influenza encephalopathy, human herpesvirus-6 (HHV-6) encephalopathy and rotavirus encephalopathy, whereas the other is based on clinical, laboratory, imaging and pathological findings of encephalopathy. With recent advances in this syndrome classification,

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many novel syndromes, such as acute necrotizing encephalopathy (ANE) [2], acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) [3] and clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS) [4], have been established.

At present, epidemiologic data on acute encephalopathy are limited. In the United States, the California Encephalitis Project has collected a large number of cases of central nervous system infection since 1988; however, this study focused primarily on encephalitis, not on encephalopathy [5]. In Japan, several attempts have previously been made to estimate the morbidity and mortality of acute encephalitis/ encephalopathy [6–10]; however, none has used syndrome classification of acute encephalopathy.

In 2010, supported by a grant from the Ministry of Health, Labour and Welfare of Japan, we started the Committee for the Research on the Etiology, Diagnosis and Treatment of Severe and Intractable Acute Encephalopathy, and conducted a nationwide survey of acute encephalopathy in Japan. This study used for the first time both classifications, pathogenic (virological) and syndrome (clinico-pathological) [1], and elucidated the relationship between viruses and syndromes.

2. Material and methods

In this study, we defined acute encephalopathy based on the following criteria: (1) acute onset of impaired consciousness after a preceding infection, and (2) exclusion of well-defined intracerebral inflammation. According to the second criterion, we excluded meningitis/ encephalitis, such as herpes simplex virus (HSV) encephalitis and acute disseminated encephalomyelitis, in which inflammatory pathology is clearly established. On the other hand, we included several conditions in which the distinction between encephalitis and encephalopathy is unclear, such as MERS [4] and acute encephalitis with refractory, repetitive partial seizures (AERRPS) [11]. We also included cases even if the respondent inadvertently failed to answer a single item.

In June 2010, we mailed a questionnaire to the heads of the Department of Pediatrics of 520 hospitals that had been qualified as institutions for training pediatric specialists by the Japanese Pediatric Society. The hospitals included all the pediatric referral centers in Japan, and were distributed all over the country.

The questionnaire items were (1) the number of cases of acute encephalopathy treated by each hospital during the last 3 years (from April 2007 to June 2010), (2) date

Table 1
Diagnostic criteria for three major syndromes.

I. *Acute necrotizing encephalopathy of childhood (ANE)*
1. Acute encephalopathy following a viral febrile disease. Rapid deterioration in the level of consciousness. Convulsions
2. No CSF pleocytosis. Increase in CSF protein commonly observed
3. CT or MRI evidence of symmetric, multifocal brain lesions. Involvement of the bilateral thalami. Lesions also common in the cerebral periventricular white matter, internal capsule, putamen, upper brain stem tegmentum and cerebellar medulla. No involvement of other CNS regions
4. Elevation of serum aminotransferases of variable degrees. No increase in blood ammonia
5. Exclusion of resembling diseases.
A. Differential diagnosis from clinical viewpoints.
Overwhelming bacterial and viral infections, and fulminant hepatits; toxic shock, hemolytic uremic syndrome and other toxin-induced diseases; Reye syndrome, hemorrhagic shock and encephalopathy syndrome, and heat stroke.
B. Differential diagnosis from radiological viewpoints.
Leigh encephalopathy and related mitochondrial cytopathies; glutaric acidemia, methylmalonic acidemia, and infantile bilateral striatal necrosis; Wernicke encephalopathy, and carbon monoxide poisoning; acute disseminated encephalomyelitis, acute hemorrhagic leucoencephalitis, other types of encephalitis and vasculitis; arterial or venous infection, and the effects of severe hypoxia or head trauma

II. *Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD)*
1. Onset with convulsion (status epilepticus convulsivus in most cases) within 24 hours from the onset of fever
2. Subsequent, transient improvement in consciousness
3. Recurrence of convulsions (clustering partial seizures in most cases) on the fourth to sixth day of illness, followed by impairment of consciousness
4. Pathogens of precedent infection: influenza virus and HHV-6, 7 in many cases
5. Variable prognosis: mild to severe psychomotor retardation. Typical cases show impaired speech and voluntariness
6. Normal MRI on the first to second day of illness
7. High signal intensity lesions in the cerebral subcortical white matter on diffusion-weighted images on the third to ninth day of illness. T2-weighted and FLAIR images may show high signal intensities along U-fibers

III. *Clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS)*
1. Onset with neuropsychiatric symptoms, such as abnormal speech and/or behavior, and impaired consciousness and convulsion, within one week after the onset of fever
2. Complete recovery without sequelae, mostly within ten days after the onset of neuropsychiatric symptoms
3. High signal intensity lesion in the splenium of corpus callosum, in the acute stage. T1 and T2 signal changes are mild
4. Lesion may involve the entire corpus callosum and the cerebral white matter in a symmetric fashion
5. Lesion disappears within a week, with neither residual signal changes nor atrophy

(year/month) and age at onset of each case, (3) sex, (4) syndrome of acute encephalopathy (e.g. ANE, AESD, MERS and others), (5) pathogen of preceding infection (e.g. influenza virus, HHV-6, unknown and others), and (6) prognosis. With regard to syndrome diagnosis (item #4), we also sent the diagnostic criteria of three major syndromes, ANE [12], AESD [13,14] and MERS [13] (Table 1), together with their typical neuroimaging findings. Diagnosis of hemorrhagic shock and encephalopathy syndrome (HSES) and other syndromes was based on previously published criteria [1,11,15]. As for prognosis (item #6), sequelae were judged as severe if the patient was unable either to walk independently or to utter meaningful words. Responses were sent back either by mail or by fax.

Statistical data were compared among the three syndromes, ANE, AESD and MERS. For numerical data (age), statistical significance was evaluated with one-way ANOVA. The homogeneity of the variances was analyzed by the Levene test; in case of P less than 0.05, pairwise comparisons were made and corrected by Bonferroni method. For categorical data (outcome), we used chi square tests with residual analysis.

This study was based on the Ethical Guideline for Epidemiological Researches published by Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare, Japan.

3. Results

3.1. Acute encephalopathy as a whole

Of the 520 hospitals, 265 (51.0%) responded. The total number of cases of acute encephalopathy was 983. The calculated annual incidence was 302 cases per year.

There were 497 males (51.0%) and 477 females (49.0%); no gender difference was noted.

Age at onset ranged from infancy to puberty. The incidence was most high in infancy and early childhood (Fig. 1). The average/standard deviation was 4.0 ± 3.7 years, and the median was 3 years.

Syndrome classification revealed that AESD was the most common (282 cases, 28.7%), followed by MERS (153 cases, 15.6%), ANE (39 cases, 4.0%), HSES (20 cases, 2.0%), limbic encephalitis (15 cases, 1.5%), Reye-like syndrome (7 cases, 0.7%), AERRPS (6 cases, 0.6%), Reye syndrome (4 cases, 0.4%) and posterior reversible encephalopathy syndrome (PRES) (4 cases, 0.4%). Thirteen cases (1.3%) had other syndromes, and 431 cases (43.8%) remained unclassified.

Among pathogenic viruses of preceding infection, influenza virus was the most common (263 cases, 26.6%), followed by HHV-6 (168 cases, 17.0%), rotavirus (40 cases, 4.0%), respiratory syncytial virus (RSV) (17 cases, 1.7%), mumps virus (9 cases, 0.9%), adenovirus (7 cases, 0.7%), HHV-7 (6 cases, 0.6%), HSV (6 cases, 0.6%), norovirus (5 cases, 0.5%), Epstein Barr virus (3 cases, 0.3%), varicella-zoster virus (3 cases, 0.3%), human parechovirus (2 cases, 0.2%) and measles virus (1 case, 0.1%). Bacterial pathogens, such as enterohemorrhagic *Escherichia coli* and *Salmonella*, were detected in 16 cases (1.6%), and *Mycoplasma pneumoniae* in 9 cases (0.9%). Concomitant infections, such as HHV-6/RSV and rotavirus/*Campylobacter jejuni* or *coli*, were found in 5 cases (0.5%). Pathogens remained unidentified in 401 cases (40.8%).

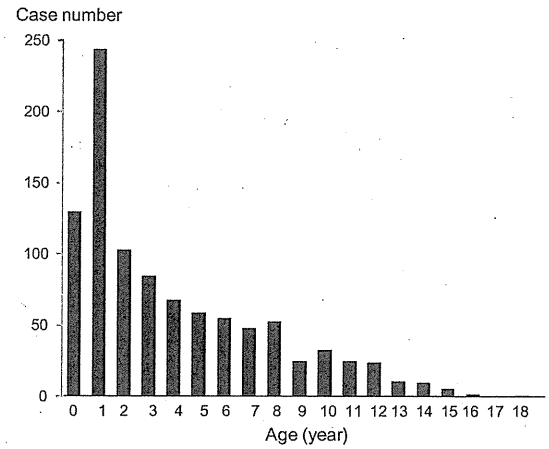

Fig. 1. Age distribution of acute encephalopathy.

The outcome of acute encephalopathy varied. Full recovery was noted in 552 cases (56.2%), mild to moderate sequelae in 218 (22.1%), severe sequelae in 133 (13.5%), and death in 55 (5.6%).

3.2. Major syndromes of acute encephalopathy

3.2.1. AESD

AESD was the most frequent syndrome (282 cases), with 114 male (40.4%) and 167 female (59.6%) patients. Age distribution showed a high incidence in infancy (average/standard deviation 1.7 ± 2.2 years, median 1 year) (Fig. 2).

Pathogens of the preceding infection were HHV-6 in 108 cases (38.2%), influenza virus in 27 (9.5%), HHV-7 in 5 (1.8%), rotavirus in 4 (1.4%) and RSV in 4 (1.4%). There were no cases of bacterial infection.

Outcome of AESD was characterized by low fatality and a high incidence of neurologic sequelae. Full recovery was noted in 81 patients (28.7%), mild to moderate sequelae in 116 (41.1%), severe sequelae in 71 (25.1%) and death in only 4 (1.4%). The ratio of patients with mild to moderate sequelae was significantly higher than for ANE ($P < 0.01$) and MERS ($P < 0.01$).

3.2.2. MERS

MERS was the second most frequent syndrome (153 cases), with 80 male (52.3%) and 69 female (45.1%)