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インフルエンザ等の呼吸器感染症に関する研究

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総括研究報告書

インフルエンザ等の呼吸器感染症に関する研究

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研究要旨
ウイルス学的にインフルエンザ関連と診断した脳炎・脳症 11 例について臨床的経過・ウイルス RNA の局在について解析した。インフルエンザ関連脳炎・脳症例の一部にウイルス血症を認め、本症はウイルス血症に引き続いた脳血管障害・虚血が主たる病態と推測された。

分担研究者氏名・所属施設名及び所属施設における職名

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教授

A．研究目的

近年、インフルエンザ罹患時の脳炎・脳症による死亡の増加が注目を集めている。本研究の目的はインフルエンザ関連の脳炎・脳症の発症のメカニズムを解明することである。

B．研究方法

1997-98 年度にウイルス学的にインフルエンザ関連と診断した脳炎・脳症 11 例について、臨床症状・経過を解析した。また、血液中、髄液中のウイルス RNA の局在の有無を RT-PCR 法にて検討した。

C．研究結果

1. 脳炎・脳症は、ワクチン歴のない小児にインフルエンザ発病早期に主に痙攣・意識障害を主要症状として発症し、約 40％に神経学的後遺症を残し、10％が死亡していた。
2. 臨床的には、急性壊死性脳症を呈したものが 1 例、ライ様症候群、Hemorrhagic shock and Encephalopathy（HSE）の経過をたどったものが 3 例であった。
3. 脳炎・脳症患者中 1 例の血中にインフルエンザ RNA を認めた。一方、コントロール群では全例陰性であった。
4. 1 例の髄液中にウイルス RNA を認めた。

D．考察

インフルエンザ関連脳炎・脳症は臨床的に脳浮腫・急性壊死性脳症、Hemorrhagic shock and Encephalopathy（HSE）などの血管障害、虚血を疑わせる病態が特徴的であった。また、一部の症例であるがウイルス血症が認められたこと、髄液中にウイルス RNA が存在しなかったことにより、本症は中枢神経系へのウイルスの侵入、増殖が主たる病態ではなく、脳血管の透過性亢進、脳虚血が本態である可能性が示唆された。

E．結論

インフルエンザ関連脳炎・脳症はウイルス血症後に引き続く、脳細胞の透過性の亢進、脳虚血が主たる病態と考えられる。

F．研究発表

1. 論文発表

Ito Y, et. al. Detection of Inflrenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-virus-associated encephalopathy, Journal of Medical Virology,in press,1999

2. 学会発表

(1) 伊藤嘉規　他、インフルエンザ関連脳炎・脳症におけるウイルス動態を中心とした病態および臨床像の検討、第 46 回日本ウイルス学会学術集会・総会、1998.10.12、東京

(2) 伊藤嘉規、他、1997-98 シーズンのインフルエンザ関連脳炎・脳症の検討、第 102 会日本小児科学会総会学術集会、1999 年、東京

G．知的所有権の取得状況

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

厚生科学研究費補助金（厚生科学特別研究事業）
分担研究報告書

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分担研究者　森島恒雄　名古屋大学　教授

研究要旨
ウイルス学的に、インフルエンザ関連と診断した脳炎・脳症 11 症例について、血中・髄液中のサイトカインを測定した。一部の症例の血中、髄液中に炎症性サイトカイン、特に IL-6 の上昇を認め、本症の病態がこれら炎症性サイトカインを介して生じる可能性が示唆された。

分担研究者氏名・所属施設名及び所属施設における職名

省略

A．研究目的

近年、インフルエンザ罹患時の脳炎・脳症による死亡の増加が注目を集めている。本研究の目的はインフルエンザ関連の脳炎・脳症の発症のメカニズムを解明することである。

B．研究方法

1997-98 年度にウイルス学的にインフルエンザ関連と診断した脳炎・脳症 11 例について血中、髄液中のサイトカイン（ TNF-α, sTN-R1,IL-1B,IL-6）を EIA 法にて測定した。

C．研究結果

1. 11 例中、6 例の血清中に何らかの炎症性サイトカインの上昇を認めた。特に IL-6 は 3 例に高値例が認められコントロール群との間に有意差を認めた。
2. 11 例中、5 例の髄液中に何らかの炎症性サイトカインの上昇を認めた。

D．考察

近年、IL-6 は血管の透過性を高め血液脳関門を破壊することが報告されている。ウイルス血症により、インフルエンザウイルスが血管内皮細胞に感染、そこで産生された炎症性サイトカインが本症の発症に直接関与している可能性がある。主任研究者（木村宏）が示した本症の臨床像、ウイルス RNA の局在のデータはこの仮説を裏づけるものである。

E．結論

インフルエンザ関連脳炎・脳症は炎症性サイトカインを介した。脳血管障害・虚血が主たる病態である可能性が示唆された。

F．研究発表

1. 論文発表
   Ito Y, et. al. Detection of Inflrenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-virus-associated encephalopathy, Journal of Medical Virology,in press,1999
2. 学会発表
   (1) 伊藤嘉規　他、インフルエンザ関連脳炎・脳症におけるウイルス動態を中心とした病態および臨床像の検討、第 46 回日本ウイルス学会学術集会・総会、1998.10.12、東京
   (2) 伊藤嘉規、他、1997-98 シーズンのインフルエンザ関連脳炎・脳症の検討、第 102 会日本小児科学会総会学術集会、1999 年、東京

G．知的所有権の取得状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

Detection of Influenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-virus-associated encephalopathy

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Shortened title; RT-PCR and Cytokines in Influenza Encephalopathy

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ABSTRACT

Eleven children with acute encephalopathy associated with an influenza virus infection were treated during the 1997-98 flu season. Reverse transcription (RT)-PCR assay was used to detect the viral genome in peripheral blood and cerebrospinal fluid (CSF) samples from these patients. The results were compared with those of control influenza patients without neurological complications. Virus RNA was detected only in the peripheral blood mononuclear cells of one patient with influenza-virus-associated encephalopathy (1 of 9; 11 %) and in the CSF of another patient (1 of 11; 9 %). RT-PCR was negative in the blood of all the controls, but the percentage of RT-PCR-positive samples in the two groups was not significantly different. Cytokines and soluble cytokine receptors in plasma and CSF were then quantified using an enzyme-linked immunosorbent assay. The CSF concentrations of soluble tumor necrosis factor receptor-1 were elevated in two patients and interleukin-6 (IL-6) was elevated in one patient with influenza-virus-associated encephalopathy. On the other hand, the plasma concentrations of IL-6 were elevated in 4 of 9 patients. The number of encephalopathy patients who had elevated plasma concentrations of IL-6 $\geq$ 100 pg/mL was significantly higher than that of controls ($p = 0.01$). In conclusion, the infrequent detection of the virus genome in the CSF and blood showed that direct invasion of the virus into the central nervous system was an uncommon event. Proinflammatory cytokines and soluble cytokine receptors may mediate the disease. The high plasma concentration of IL-6 could be an indicator of the progression to encephalopathy.

KEY WORDS

Influenza virus, encephalopathy, RT-PCR, interleukin 6

INTRODUCTION

Infection with influenza viruses can produce a spectrum of clinical responses ranging from a febrile upper respiratory illness to central nervous system (CNS) involvement with significant mortality. After the first human influenza virus was isolated in 1933, several papers reported examples of influenza-associated encephalopathy. Two specific types of acute encephalopathy are reported to accompany influenza infection: Reye syndrome and influenza-associated encephalopathy. Reye syndrome, which is a neurologic and metabolic disease with hepatic dysfunction and fatty accumulation in the viscera, often follows viral infections and the use of salicylate [Balistreri, 1996].

Influenza-associated encephalopathy, which occurs at the height of illness and may be fatal, has been described by many authors [Dunbar et al., 1958; Flewett et al., 1958; McConkey et al., 1958; Delorme et al., 1979; Protheroe et al., 1991; Murphy et al., 1996]. The cerebrospinal fluid (CSF) is usually normal, the brain shows severe congestion at autopsy, and histological changes are minimal [Murphy et al., 1996]. The pathogenesis of this CNS symptomatology is, however, unclear. In regards to the viral pathogenesis, one explanation is that CNS complications may be caused by hematogenous transmission of the virus to the CNS, although the existence of viremia is under dispute and isolation of the influenza virus from CSF is rare [Stanley et al., 1969; Lehmann et al., 1971; Mori et al., 1997; Tsuruoka et al., 1997].

In the 1997-98 flu season, 11 children with acute influenza-virus-associated encephalopathy were treated. Reverse transcription (RT)-PCR assay was used to detect the viral genome in

peripheral blood and CSF samples. Several cytokines and soluble cytokine receptors were quantified in samples from encephalopathy patients. The presence of tumor necrosis factor-α (TNF-α), soluble tumor necrosis factor receptor 1 (sTNF-R1), interleukin-1β (IL-1β), and IL-6 in CSF samples is important for predicting the clinical outcome and diagnosing encephalitis/encephalopathy [Ichiyama et al., 1996a, 1998]. However, little is known about the levels of these cytokines in plasma and CSF from patients with influenza-virus-associated encephalopathy. Study of the dynamics of these cytokines may improve understanding of the mechanisms of influenza-virus-associated encephalopathy.

MATERIALS AND METHODS

Patients and Controls

Eleven consecutive patients, aged 2 to 13 years (7 boys, 4 girls; mean age: 5.7 years), who were diagnosed with influenza-virus-associated encephalopathy between January and February 1998, were investigated. The clinical data for these patients are summarized in Table 1. The level of consciousness was assessed using the Glasgow Coma Scale [Teasdale et al., 1974; Reilly et al., 1988]. Influenza-virus-associated encephalopathy was defined as follows: (1) The patient had a preceding upper respiratory tract infection and an altered level of consciousness that could not be explained by other identifiable causes. (2) Reye syndrome according to the case definition of the Center for Disease Control and Prevention (U.S.A.) [Center for Infectious Diseases, 1991] was excluded. (3) Influenza virus RNA was detected in throat swabs with the RT-PCR assay. The serum hemagglutinin inhibition

titer of antibody to H3N2 virus increased significantly in all 9 patients in which it was measured, at least fourfold from acute to convalescent titers.

Twenty nine control patients aged 1 to 15 years (13 boys, 16 girls; mean age: 3.8 years) with influenza virus infections without any neurological complications were also studied. In all the control subjects, the diagnosis of an influenza virus infection was also confirmed by the detection of viral RNA in throat swabs.

Samples

Peripheral blood samples from the patients and controls were collected in standard blood tubes containing EDTA. Plasma, peripheral blood mononuclear cell (PBMC), and erythrocyte fractions were isolated from 1 mL of whole blood by Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density centrifugation at 400 X g for 30 min at room temperature. The PBMC and erythrocyte fractions were washed twice with PBS, resuspended in 200 μL of PBS, and stored at -70°C until use. CSF was obtained from patients with influenza-virus-associated encephalopathy and stored at -70°C.

RT-nested PCR

For PCR aimed at the NS gene, sense primer NS3 (GGTGATGCCCCATTCCTTGA; positions 108 to 127) and antisense primer NS4 (ATTTCGCCAACAATTGCTCC; positions 486 to 505) were used in the first round. Primers NS1 (GAGGCACTTAAAATGACCAT; positions 249 to 268) and NS2 (CTCTTCGGTGAAAGCCCTTAG; positions 465 to 485) were used in the nested PCR reaction. These oligonucleotides were designed from the highly conserved region of the influenza A/PR/8/34 NS gene sequence [Buonagrio et al., 1986].

RNA was extracted from each sample using a QIAamp viral RNA kit (QIAGEN, Hilden, Germany), using a silica-gel-based membrane that binds RNA. The RNA extracted from 200 μL of each sample was eluted in 50 μL RNase-free water. Ten microliters of this solution were used for cDNA synthesis immediately after denaturation for 2 min at 80°C. The reaction buffer (final concentrations, 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl2), NS3 sense primer (25 pmol), deoxynucleoside triphosphates (0.5 mM final concentration), 200U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Rockville, U.S.A.), and dithiothreitol (50 mM final concentration) were added to a final volume of 20 μL. After incubation at 37°C for 60 min, 5 μL of this solution was added to 45 μL of PCR mixture containing NS3 and NS4 primers (25 pmol each), 1.5 U of Taq DNA polymerase (TaKaRa Taq; Takara Syuzou, Otsu, Japan), and the same reaction buffer as used in the RT reaction. Amplification was carried out in a TP-240 thermal cycler (Takara Syuzou). The PCR program consisted of a 1 min preincubation at 94 °C followed by 30 cycles of 1 min at 94°C and 20 sec at 62°C. Nested PCR was performed after transferring 1 μL of the first round PCR product into a new PCR reaction mixture containing the nested primers under the same conditions. The nested amplification product, which was expected to yield a 237 base-pair sequence, was analyzed by electrophoresis through 1.2% agarose in a Tris-acetate-EDTA gel stained with ethidium bromide. Since the sequences of the designed primers are highly conserved, both influenza A and influenza B viruses were detectable (Data not shown).

Synthesis of Positive Control RNA

A first round PCR fragment, consisting of nucleotides 108 to 505 of the NS gene, was cloned into the pGEM-T plasmid (Promega). RNA transcripts were synthesized from the purified recombinant plasmid with T7 RNA polymerase (the Riboprobe in vitro transcription system; Promega) and serially diluted in diethyl pyrocarbonate-treated water. Tenfold dilutions were tested by RT-PCR, and the detection limit was established reproducibly.

Enzyme-linked Immunosorbent Assay for Cytokines and Soluble Cytokine Receptors

The concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 were determined with commercial sandwich-type enzyme-linked immunosorbent assay (ELISA) kits (IL-1β kit; Genzyme, Cambridge, U.S.A.: TNF-α, sTNF-R1, and IL-6 kits; R&D Systems, Minneapolis, U.S.A.). These assays were performed according to the supplier's instructions. Sample values were determined from a standard curve.

Statistical Analysis

Data were analyzed using Fisher's exact test. A level of $p < .05$ was considered significant.

RESULTS

Sensitivity of RT-PCR

To determine the sensitivity of our RT-PCR assay, dilutions of synthesized RNA transcripts of the NS gene were prepared (Materials and Methods) and used for the RT-PCR assay. We were able to detect a minimum of 3 copies per 50 μL PCR reaction mixture (Fig. 1).

Detection of Influenza Virus RNA

RT-PCR was performed using blood samples (plasma, PBMC, erythrocytes) from the patients and controls, and CSF samples from the patients (Table 2). Viral RNA was only detected in the PBMC of one patient with influenza-virus-associated encephalopathy [1 (patient 9) of 9; 11 %] and in the CSF of another patient [1 (patient 8) of 11; 9 %]. Viral RNA was not detected in plasma or erythrocytes from any of the patients. RT-PCR was also negative with all the blood samples from the controls. The percentages of RT-PCR positive blood samples in the two groups were not significantly different. The detection of viral RNA was not associated with any clinical features or the outcome, although the number of positive patients was small.

The Concentrations of Cytokines and Soluble Cytokine Receptors

The levels of TNF-α, sTNF-R1, IL-1β, and IL-6 in the CSF of the patients with influenza-virus-associated encephalopathy are shown in Table 3. The concentrations of TNF-α and IL-1β in the CSF were all below the detection limits. The CSF concentrations of sTNF-R1 and IL-6 were elevated in 2 and 1 patients, respectively, out of 7 with influenza-virus-associated encephalopathy.

The levels of TNF-α, sTNF-R1, IL-1β, and IL-6 in the plasma of the patients with encephalopathy are shown in Table 4. The plasma TNF-α concentrations were all below the detection limits. In the 9 patients with influenza-virus-associated encephalopathy, the plasma concentrations of sTNF-R1, IL-1β, and IL-6 (particularly IL-6 $\geq$ 100 pg/mL in 3) were elevated in 2, 2,

and 4 patients, respectively. The number of influenza-virus-associated encephalopathy patients who had elevated concentrations of IL-6 ≥ 100 pg/mL was significantly higher than that of the controls ($p = 0.01$) (Table 5). There were no significant differences in the numbers of patients and controls with elevated concentrations of TNF-α, sTNF-R1, or IL-1β (Table 5).

The concentrations of cytokines and soluble cytokine receptors in the CSF and plasma were not associated with any clinical features in the encephalopathy patients. In terms of mortality and morbidity, two patients who had cytokines in both CSF and plasma recovered without sequelae (patients 3 and 4).

DISCUSSION

Viremia is unusual in influenza virus infection [Murphy et al., 1996], although the virus is occasionally isolated from the blood [Stanley et al., 1969, Lehmann et al., 1971]. Even when the RT-PCR assay is used, influenza RNA is only detected occasionally in blood samples from influenza patients [Mori et al., 1997, Tsuruoka et al., 1997]. In our study, viral RNA was detected infrequently in blood from encephalopathy patients and never in blood from the controls. Viremia may be as rare in patients with influenza-virus-associated encephalopathy as it is in patients with influenza infection. Alternatively, the virus might be present in low titers in the blood.

Human influenza A viruses are reported to be neurovirulent in mouse models. Mice infected with influenza A viruses by intracerebral inoculation developed a meningoencephalitic condition

[Nakajima et al., 1980, Sugiura et al., 1980, Takahashi et al., 1995]. Previously, we reported that using PCR assay to detect the herpes simplex virus genome in CSF is useful for virological assessment of patients with herpes simplex virus encephalitis [Kimura et al., 1991, 1992; Ando et al., 1993]. If influenza virus replicates in the brain tissue in a similar way to herpes simplex, then RT-PCR assay should also be a useful tool for analyzing influenza-associated-encephalopathy. A recent Japanese study frequently detected virus RNA in the CSF from patients with influenza-associated-encephalopathy [Fujimoto et al., 1998]. In that study, the RT-PCR assay of 5 of 7 patients seen in the 1996-97 flu season was positive. The authors didn't perform RT-PCR on blood samples. In the present study, we established an RT-PCR assay to detect influenza virus RNA. Using this highly sensitive method, we found that the RT-PCR assay was positive in only 1 of 11 CSF samples from patients with influenza-virus-associated encephalopathy. This result shows that although viral replication may occur in the CNS, it is an uncommon event. It is not known why the frequency of detection of viral RNA differed in the two studies. One possibility is that the rate of CNS invasion differs according to the epidemic virus, although we have little information regarding to the respective capacity of 1996-97 and 1997-98 season viruses to induce encephalopathy.

Many cytokines and soluble cytokine receptors are considered important mediators of inflammatory responses, and their levels increase in CSF or plasma in infectious inflammatory disorders of the CNS, primarily meningitis [Mustafa et al., 1989; Chavanet et al., 1992; Glimåker et al., 1993; López-Cortés et al., 1993; Aurelius et al., 1994; Ichiyama et al., 1996a, 1996b, 1997, 1998].

We also reported previously that elevation of TNF-α, IL-1β, and IL-6 in the CSF indicates acute encephalitis/encephalopathy, rather than febrile convulsions mimicking acute encephalitis/encephalopathy [Ichiyama et al., 1998]. Previous studies showed that sTNF-R1 is the natural homeostatic regulator of the action of TNF-α, and that the level of sTNF-R1 is a better indication of the true biological activity of TNF-α than the level of TNF-α itself [Duncombe et al., 1988; Englemann et al., 1990]. In the present study, the CSF concentrations of sTNF-R1 and IL-6 were elevated in 2 and 1 of 7 patients, respectively, with influenza-virus-associated encephalopathy. It is not clear why sTNF-R1 and IL-6 were not always detected in the CSF. The inflammation of the CNS may be mild, so that inflammatory cytokines cannot be detected. Alternatively, influenza-virus-associated encephalopathy may have a different pathogenesis. In the influenza B virus mouse model of Reye syndrome, intravenous inoculation of the virus caused a non-permissive viral infection of vascular endothelial cells of the brain and damage to the blood-brain barrier that resulted in acute encephalopathy without inflammation [Davis et al., 1990]. In an autopsy case of human herpesvirus 6 encephalopathy, human herpesvirus 6 viral antigens were detected only in the vascular endothelium of the brain and no inflammation was observed [Ueda et al., 1996]. These observations suggest that vascular endothelial infection is part of the pathogenesis of acute encephalopathy. Toxic factors and metabolic disorders, including hereditary enzymatic deficiency, are other possibilities.

The number of influenza-virus-associated encephalopathy patients who had elevated concentrations of IL-6 $\geq$ 100 pg/mL in plasma was significantly higher than that in the controls in our

study. Monocytes and lymphocytes produce IL-6; however, it is particularly interesting that IL-6 is also produced by the vascular endothelium. IL-6 plays an important role in host responses to infection and induces hepatic protein synthesis, including C-reactive protein and fibrinogen, during the acute phase response [Heinrich et al., 1990]. Recently, it was reported that IL-6 affected the permeability of the blood-brain barrier in rats [Saija et al., 1995, Farkas et al., 1998]. In human neonates, IL-6 is thought to play a role in hypoxic-ischemic brain damage [Martín -Ancel et al., 1997]. It is possible that the systemic reaction to IL-6 contributes to the development of the influenza-virus-associated encephalopathy. Previous studies have described how IL-6 plasma concentrations are useful in the early diagnosis of neonatal infection [Messer et al., 1996, Panero et al., 1997]. Our results suggest that IL-6 plasma concentrations might also be useful in differentiating influenza-virus-associated encephalopathy.

In conclusion, the infrequent detection of the viral genome in CSF and blood indicates that direct invasion of the influenza virus into the CNS is an uncommon event, and suggests that systemic cytokines or vascular involvement may be indirectly responsible for the encephalopathy. A high plasma concentration of IL-6 may indicate progression to encephalopathy. However, the precise mechanism of the illness remains unknown. Further studies should explore the disease mechanism and the clinical applications of these observations.

ACKNOWLEDGEMENTS

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