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Detection of Influenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-Virus-Associated Encephalopathy

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Eleven children with acute encephalopathy associated with an influenza virus infection were treated during the 1997–1998 influenza season. Reverse transcription-polymerase chain reaction (RT-PCR) assay was used to detect the viral genome in peripheral blood and cerebrospinal fluid (CSF) samples. The results were compared with those of control influenza patients without neurological complications. Viral 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 four of nine patients. The number of encephalopathy patients who had elevated plasma concentrations of IL-6 100 pg/ml was significantly higher than that of controls ($P = .01$). In conclusion, the infrequent detection of the viral 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. ***J. Med. Virol. 58:420–425, 1999.***

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 examples of influenza-associated encephalopathy have been reported. 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 investigators [Dunbar et al., 1958; Flewett and Hoult, 1958; McConkey et al., 1958; Delorme and Middleton, 1979; Protheroe and Mellor, 1991; Murphy and Webster, 1996]. The cerebrospinal fluid (CSF) is usually normal, the brain shows severe congestion at autopsy, and histological changes are minimal [Murphy and Webster, 1996]. The pathogenesis of this CNS syndrome 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 disputed and isolation of the in-

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TABLE I. Clinical Features of Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	Age (years)/ Sex	GCS	Convulsion	Cerebrospinal fluid		Serum			Mortality and morbidity
				Cell count (/μl)	Protein (mg/dl)	AST (IU/L)	ALT (IU/L)	NH_3 (μg/dl)	
1	2/F	11	Yes	2	24	92	24	18	Recovery
2	2/M	12	Yes	15	27	49	15	34	Recovery
3	2/M	13	Yes	0	13	26586	13879	74	Recovery
4	2/M	12	Yes	6	21	56	41	53	Recovery
5	3/M	3	Yes	0	57	18088	10472	50	Sequelae
6	5/M	3	Yes	6	20	1276	1667	37	Sequelae
7	6/F	3	Yes	NA	NA	32	14	NA	Recovery
8	6/F	11	No	0	10	39	17	NA	Sequelae
9	11/M	11	No	NA	NA	200	72	NA	Sequelae
10	11/M	11	Yes	3	23	35	13	21	Recovery
11	13/F	3	Yes	NA	NA	10510	3160	NA	Death

GCS, Glasgow Coma Scale; AST, aspartate aminotransferase; ALT, alanine aminotransferase; F, female; M, male; NA, not applicable.

fluenza virus from CSF is rare [Stanley and Jackson, 1969; Lehmann and Gust, 1971; Mori et al., 1997; Tsuruoka et al., 1997].

In the 1997–1998 flu season, 11 children with acute influenza-virus-associated encephalopathy were treated. Reverse transcription-polymerase chain reaction (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 2–13 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 I. The level of consciousness was assessed using the Glasgow Coma Scale [Teasdale and Jennett, 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 1–15 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 patients, 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 ethylenediamine tetraacetic acid (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 × *g* for 30 min at room temperature. The PBMC and erythrocyte fractions were washed twice with phosphate-buffered saline (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 108–127) and antisense primer NS4 (ATTTCGCCAACAATTGCTCC; positions 486–505) were used in the first round. Primers NS1 (GAGGCACTTAAAATGACCAT; positions 249–268) and NS2 (CTCTTCGGTGAAAGCCCTTAG; positions 465–485) 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 [Buonagurio 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 $MgCl_2$), NS3 sense primer (25 pmol), deoxynucleoside triphosphates (0.5 mM final

concentration), 200 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Rockville, MD), 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 were 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. Because 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 108–505 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 diluted serially in diethyl pyrocarbonate-treated water. Ten-fold 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, MA; TNF-α, sTNF-R1, and IL-6 kits, R&D Systems, Minneapolis, MN). These assays were carried out 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. A minimum of three copies per 50 μl PCR reaction mixture could be detected (Fig. 1).

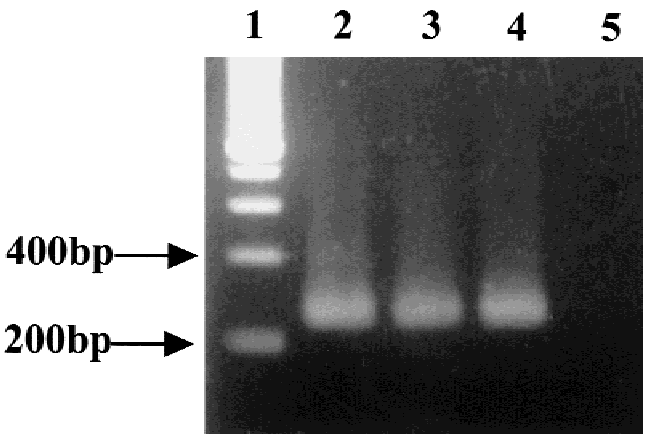

Fig. 1. Sensitivity of the reverse transcription-polymerase chain reaction (RT-PCR) in detecting influenza virus NS gene. **Lane 1**, 200 bp DNA marker ladder; lanes **2–4**, 3×10^2, 3×10^1, 3 copies of NS gene, respectively; **lane 5**, no template control.

Detection of Influenza Virus RNA

RT-PCR was carried out using blood samples (plasma, PBMC, erythrocytes) from the patients and controls, and CSF samples from the patients (Table II). Viral RNA was detected only in the PBMCs 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.

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 III. 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 two and one patients, respectively, out of seven 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 IV. The plasma TNF-α concentrations were all below the detection limits. In the nine patients with influenza-virus-associated encephalopathy, the plasma concentrations of sTNF-R1, IL-1β, and IL-6 (particularly IL-6 ≥ 100 pg/ml in three patients) were elevated in two, two, and four 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 ($P = .01$) (Table V). There were no significant differences in the numbers of patients and controls with el-

TABLE II. Results of RT-PCR in Patients With Influenza-Virus-Associated Encephalopathy

Samples	Patients	Controls
Throat swab	9/9	29/29
Plasma	0/11	0/29
PBMC	1/9	0/29
Erythrocytes	0/9	0/29
CSF	1/11	ND

RT-PCR, reverse transcription-polymerase chain reaction; PBMC, peripheral blood mononuclear cells; CSF, cerebrospinal fluid; ND, not done.

TABLE III. Cerebrospinal Fluid Concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 in Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	TNF-α (pg/ml)	sTNF-R1 (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
1	NA	NA	NA	NA
2	NA	NA	NA	NA
3	<15	1196	<4	324[b]
4	<15	2934	NA	<31.2
5	<15	1848	<4	<31.2
6	NA	NA	NA	NA
7	<15	555	<4	<31.2
8	<15	433	<4	<31.2
9	<15	553	<4	<31.2
10	<15	635	<4	<31.2
11	NA	NA	NA	NA
Normal range	<15	836 ± 402[a]	<4	<31.2

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin; NA, not applicable.
[a]Mean ± SD.
[b]Underscores represent the level considered abnormal.

TABLE IV. Plasma Concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 in Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	TNF-α (pg/ml)	sTNF-R1 (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
1	NA	NA	NA	NA
2	NA	NA	NA	NA
3	<31.2	2232	<8	860[b]
4	<31.2	810	30.2	18.2
5	<31.2	702	<8	<12.5
6	NA	426	<8	<12.5
7	<31.2	760	<8	<12.5
8	<31.2	869	<8	100
9	<31.2	>5000	<8	1295
10	<31.2	745	<8	<12.5
11	<31.2	270	21.1	<12.5
Normal range	<15.6	1020 ± 495[a]	<4	<12.5

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin; NA, not applicable.
[a]Mean ± SD.
[b]Underscores represent the level considered abnormal.

TABLE V. Comparison of the Percentage of Patients Exhibiting Plasma Cytokines

Cytokines (pg/ml)	Patients (%) (n = 9)	Controls (%) (n = 29)	P
TNF-α	0 (0)	0 (0)	1.00
sTNF-R1	2 (22)	1 (3)	.13
IL-1β	2 (22)	2 (7)	.23
IL-6	4 (44)	12 (41)	.58
IL-6 (≥100)	3 (30)	0 (0)	.01

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin.

evated concentrations of TNF-α, sTNF-R1, or IL-1β (Table V).

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 and Webster, 1996], although the virus is occasionally isolated from the blood [Stanley and Jackson, 1969; Lehmann and Gust, 1971]. Even when the RT-PCR assay is used, influenza RNA is detected only 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 patients with encephalopathy 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 and Sugiura, 1980; Sugiura and Ueda, 1980, Takahashi and Yamada, 1995]. Previously, PCR assay for detection of the herpes simplex virus genome in CSF was shown to be 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 detected viral RNA frequently in the CSF from patients with influenza-associated-encephalopathy [Fujimoto et al., 1998]. In that study, the RT-PCR assay of five of seven patients seen in the 1996–1997 influenza season was positive. RT-PCR was not undertaken on blood samples. In the present study, we established an RT-PCR assay to detect influenza virus RNA. Using this highly sensitive method, it was 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 re-

garding to the respective capacity of 1996–1997 and 1997–1998 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 during 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 and Brenner, 1988; Englemann et al., 1990]. In the present study, the CSF concentrations of sTNF-R1 and IL-6 were elevated in two and one of seven 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 nonpermissive 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.

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