with RLS (11.4 7 4.8), PD without RLS (7.0 7 3.5), and the controls (6.0 7 4.0; F2,295 = 12.29, $P < 0.0005$). Post hoc tests revealed that PD patients with RLS showed a higher PSQI score than both PD patients without RLS ($P < 0.0005$) and control subjects ($P < 0.0005$). The PSQI score for PD patients without RLS was not statistically different from that of the controls.

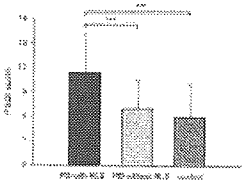

Figure 1. Pittsburgh Sleep Quality Index (PSQI) scores in Parkinson's disease (PD) with restless legs syndrome (RLS), PD without RLS, and controls. Comparisons were by analysis of covariance with a post hoc test (F2,295 = 12.29, ***$P < 0.0005$). Scores are means 7 SE.
[Normal View 11K | Magnified View 31K]

Of the background variables (Table 1), neither gender distribution nor length of PD morbidity was statistically different between the PD groups with and without RLS. However, as a group, PD patients with RLS were younger, both at the time of investigation and at the onset of PD, than PD patients without RLS. When the number of patients with young-onset PD in which symptoms of PD occurred under 40 years of age were compared between the two groups, the value was significantly higher for PD with RLS (n = 6, 30%) compared with PD without RLS (n = 8, 5.5%; $P < 0.01$, χ^2 test). Neither Hoehn and Yahr grade nor the amounts of the drugs used for the treatment of PD differed between the two PD groups; nor did the number of patients showing symptoms of reduced dopaminergic drug effectiveness differ between the two groups. Moreover, judging from the clinical interviews, RLS symptoms in PD patients did not appear to correlate with the symptoms of reduced dopaminergic drug effectiveness in all the PD patients with RLS. Serum iron and ferritin values did not differ between the PD groups with and without RLS. Only 1 PD patient with RLS (5%) showed a serum ferritin level lower than the recommended cutoff value of 50 ng/ml, under which iron replacement therapy for RLS is recommended.[22]

Table 1. Comparison of clinical background variables between PD with RLS and without RLS

	PD with RLS (n = 20)	PD without RLS (n = 145)	*P*
Age at investigation (yr)	59.7 7 2.7	70.1 7 0.8	0.0001
Gender (M/F)	8/12	59/86	n.s.
Length of PD morbidity (yr)	10.1 7 1.8	10.3 7 0.6	n.s.
Age at onset of PD (yr)	49.7 7 3.7	59.2 7 0.9	0.05
Young-onset PD (+/-)	6/14	8/138	0.005
Hoehn and Yahr grade	2.6 7 0.2	2.9 7 0.1	n.s.
Amount of drugs for the treatment of PD			
Levodopa (mg/day)	510 7 151	337 7 11	n.s.
Dopamine agonist (mg/day)[a]	4.7 7 1.0	6.0 7 0.5	n.s.
Trihexyphenidyl (mg/day)	0.5 7 0.3	0.7 7 0.1	n.s.
Droxydopa (mg/day)	90 7 39	63 7 15	n.s.
Amantadine (mg/day)	30 7 14	33.7 7 5.3	n.s.
Selegiline (mg/day)	2.25 7 0.6	2.19 7 0.2	n.s.
Serum ferritin (ng/ml)	79.0 7 7.7	76.9 7 3.9	n.s.
Serum Fe (μg/dl)	69.9 7 6.0	70.4 7 1.7	n.s.

[a] The amount of dopaminergic agonists was converted into bromocriptine equivalents.
Values are expressed as mean 7 SE.
RLS, restless legs syndrome; PD, Parkinson's disease; n.s., not significant.

DISCUSSION

Through detailed interviews and examinations, the prevalence of RLS in our control subjects (2.3%) was estimated to be much lower than in reports on the general Caucasian population.[2-4] This finding corroborates the finding that RLS was less frequent in a southeast Asian population.[5] However, the prevalence of RLS in our PD patients was much higher than that in healthy controls, despite its being slightly smaller than the value for prevalence in the United States, where 20.3% of the PD patients had RLS symptoms.[13] Our results support an etiological link between RLS and PD

beyond any gender or ethnic difference. Although the cause of the difference between the results of our study and the study in Singapore[16] is unknown, differences in culture as well as both social and educational conditions should be taken into consideration.

Although some previous reports indicated that sleep disturbance is significantly more frequent in PD patients than in the healthy population,[23][24] PSQI score was not statistically different between PD without RLS and controls in our results. The reason for this finding is unclear. However, our control subjects consisted mostly of family members taking care of patients with neurological disorders, and sleep disturbance had been reported to be more prevalent in this population than in the general population.[23][24] One possible explanation for the PSQI finding described above is that sample bias might mask the difference in the prevalence of sleep disturbance between controls and PD patients without RLS.

Our most striking finding was that PD patients with RLS showed a significantly higher PSQI score than PD patients without RLS. This finding is consistent with that reported by Krishnan et al.,[15] who found that 90% of PD patients with RLS showed delayed sleep onset. These findings suggest that sleep disturbance becomes prominent when a PD patient is affected with RLS. We speculate that sleep disturbance is more likely to become pronounced after the occurrence of RLS in PD patients who have potential sleep disturbance, such as decreased sleep efficiency, increased waking time after sleep onset, and an increased number of nocturnal awakenings.[25]

Of 20 PD patients with RLS, 7 reported an asymmetrical appearance of RLS symptoms. However, also consistent with the report by Krishnan and associates,[15] laterality of RLS symptoms had no correlation with that of PD symptoms. PD patients with RLS thought their RLS symptoms were sensory and motor symptoms associated with PD. These symptoms were first attributed to RLS by the investigators.

Moreover, only three patients requested treatment for RLS at the interviews. This finding emphasize the mild RLS severity in PD and corroborates the report of RLS symptoms in PD being often transient and irregular.[13] However, at the time of investigation, all the PD patients with RLS were already medicated with dopaminergic drugs, which are well known to suppress RLS.[12] This bias should be taken into consideration when analyzing the severity of RLS in PD patients, and future study on drug naive PD patients associated with RLS is required to draw a conclusion.

The percentage of PD patients with RLS who reported having a positive family history of RLS was smaller than the previously reported percentage in idiopathic RLS.[13] This finding is consistent with previous reports,[15] suggesting that RLS in PD patients does not occur on a genetic basis.

The findings of previous studies raised the possibility that a low level of serum ferritin could be related with the occurrence of RLS in PD.[13][15] Considering that iron has a role in both biosynthesis and transmission of monoamines, particularly dopamine,[26] this hypothesis appears reasonable. However, in our study, there was no difference in the value of serum iron between PD patients with and without RLS, suggesting that dysfunction of the central dopaminergic system itself due to PD, rather than iron deficiency, might be responsible for the occurrence of RLS in this patient group. Moreover, consistent with a report by Ondo and coworkers,[13] our results did not reveal a difference in either the symptomatic characteristics of PD or the amount of drugs used for the treatment of the two PD groups. However, the number of young-onset PD patients was significantly higher in the PD group with RLS than in the PD group without RLS. This finding may explain the finding that age, both at the investigation and at the onset of PD, was younger in PD with RLS than PD without RLS. Although no pathological relationship between young-onset PD and RLS is known, our findings are quite different from the previous reports in which PD patients affected with RLS showed an older age at investigation,[15] suggesting that the prevalence of RLS secondary to PD did not seem to relate to advancing age as it does in late-onset RLS cases in the general population. Future study on a larger sample is necessary to confirm this finding.

Our study suffers from certain limitations. Referral bias might exist in this study, and our results, therefore, may not represent the true prevalence of RLS in the general population. A large part of the clinical information was based on interview of patients included in the study, perhaps leading to recall bias with regard to RLS symptoms. However, our results do indicate that the frequency of RLS is higher in PD patients than in controls in Japan. This study emphasizes the importance of diagnosing RLS in patients with PD to aid the management of sleep disturbance.

REFERENCES

1 Ekbom KA. Restless legs syndrome. *Neurology* 1960; **10**: 863-873. Links

2 Rothdach AJ, Trenkwalder C, Haberstock J, Keil U, Berger K. Prevalence and risk factors of RLS in an elderly populations: the MEMO Study. Memory and morbidity in Augsburg elderly. *Neurology* 2000; **54**: 1064-1068. Links

3 Lavigne GJ, Montplaisir JY. Restless legs syndrome and sleep bruxism: prevalence and association among Canadians. *Sleep* 1994; **17**: 739-743. Links

4 Chokroverty S, Jankovic J. Restless legs syndrome: a disease in search of identity. *Neurology* 1999; **52**: 907-910. Links

5 Tan EK, Seah A, See SJ, et al. Restless legs syndrome in an Asian population: a study in Singapore. *Mov Disord*

2001; **16**: 577-579. Links

6 Ohayon MM, Roth T. Prevalence of restless legs syndrome and periodic limb movement disorder in the general population. *J Psychosom Res* 2002; **53**: 547-554. Links

7 Desautels A, Turecki G, Montplaisir J, et al. Identification of a major susceptibility locus for restless legs syndrome on chromosome 12q. *Am J Hum Genet* 2001; **69**: 1266-1270. Links

8 Zucconi M, Ferini-Strambi L. Epidemiology and clinical findings of restless legs syndrome. *Sleep Med* 2004; **5**: 293-299. Links

9 Gigli GL, Adorati M, Dolso P, et al. Restless legs syndrome in end-stage renal disease. *Sleep Med* 2004; **5**: 309-315. Links

10 Manconi M, Govoni V, De Vito A, et al. Pregnancy as a risk factor for restless legs syndrome. *Sleep Med* 2004; **5**: 305-308. Links

11 Salih AM, Gray RE, Mills KR, Webley M. A clinical, serological and neurophysiological study of restless legs syndrome in rheumatoid arthritis. *Br J Rheumatol* 1994; **33**: 60-63. Links

12 Littner MR, Kushida C, Anderson WM, et al. Practice parameters for the dopaminergic treatment of restless legs syndrome and periodic limb movement disorder. *Sleep* 2004; **27**; 557-559. Links

13 Ondo WG, Vuong KD, Jankovic J. Exploring the relationship between Parkinson disease and restless leg syndrome. *Arch Neurol* 2002; **59**: 421-424. Links

14 Garcia-Borreguero D, Odin P, Serrano C. Restless legs syndrome and PD: a review of the evidence for a possible association. *Neurology* 2003; **61**: S49-S55. Links

15 Krishnan PR, Bhatia M, Behari M. Restless leg syndrome in Parkinson's disease: a case-controlled study. *Mov Disord* 2003; **18**: 181-185. Links

16 Tan EK, Lum SY, Wong MC. Restless legs syndrome in Parkinson's disease. *J Neurol Sci* 2002; **196**: 33-36. Links

17 Gelb DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 1999; **56**: 33-39. Links

18 Doi Y, Minowa M, Uchiyama M, et al. Psychometric assessment of subjective sleep quality using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI) in psychiatric disordered and control subjects. *Psychiatry Research* 2000; **97**: 165-172. Links

19 Allen RP, Picchietti D, Hening WA, et al; Restless Legs Syndrome Diagnosis and Epidemiology workshop at the National Institutes of Health; International Restless Legs Syndrome Study Group. Restless legs syndrome: diagnostic criteria, special considerations, and epidemiology. A report from the restless legs syndrome diagnosis and epidemiology workshop at the National Institutes of Health. *Sleep Med* 2003; **4**: 101-119. Links

20 Hasegawa K. [The new Parkinson's disease drugs.] *Nippon Rinsho* 2000; **58**: 2066-2071.In Japanese. Links

21 The International Restless Legs Syndrome Study Group. Validation of the International Restless Legs Syndrome Study Group rating scale for restless leg syndrome. *Sleep Med* 2003; **4**: 121-132. Links

22 Sun ER, Chen CA, Ho G, Earley CJ, Allen RP. Iron and the restless legs syndrome. *Sleep* 1998; **21**: 371-377. Links

23 Pal PK, Thennarasu K, Fleming J, et al. Nocturnal sleep disturbance and daytime dysfunction in patients with Parkinson's disease and in their caregivers. *Parkinsonism Relat Disord* 2004; **10**: 157-168. Links

24 Smith MC, Ellgring H, Oertel WH. Sleep disturbances in Parkinson's disease patients and spouse. *J Am Geriatr Soc* 1997; **45**: 194-199. Links

25 Factor SA, McAlarney T, Sanchez-Ramos JR, Weiner WJ. Sleep disorders and sleep effect in Parkinson's disease. *Mov Disord* 1990; **5**: 280-285. Links

26 Yodium M, Ben-Schacher D, Ashkenazi R, et al. Brain iron and dopamine receptor function. In: Mandel P , De Feudis F , editors. *CNS receptors - from molecular pharmacology to behavior.* New York: Raven Press; 1983. p 309-321.

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Original Article

Apoptosis under hypercytokinemia is a possible pathogenesis in influenza-associated encephalopathy

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Abstract

Background: Influenza-associated encephalopathy is reported to be frequent in Japan and East Asia. No evaluating markers except interleukin (IL)-6 and tumor necrosis factor (TNF)-α and no likely pathological mechanism for the disease have yet been elucidated.

Methods: In this study, influenza-associated encephalopathy was defined by clinical symptoms, and the use of an anti-influenza antibody test and/or influenza antigen detection kits, as well as computed tomography and/or magnetic resonance imaging. The levels of proinflammatory cytokines, acute phase proteins, endothelial markers and cytochrome c were compared in sera from 11 patients with and 42 without encephalopathy.

Results: Cytochrome c concentration in sera from patients with encephalopathy was markedly increased compared with that from patients without encephalopathy and normal controls. Although levels of several other proinflammatory cytokines and acute phase proteins such as TNF-α and IL-8 were also elevated in patients with influenza virus infection, the difference between those with and without encephalopathy, though significant, was less dramatic. The mean serum concentration of cytochrome c in 11 patients with encephalopathy, consisting of four deceased, four with and three without residual central nervous system sequelae, was 26.7 ± 19.5 ng/mL on admission. In contrast, cytochrome c levels in 42 patients without encephalopathy were 0.3 ± 0.7 ng/mL.

Conclusion: The present results indicate that cytochrome c is a useful marker to follow patients with influenza-associated encephalopathy and suggest that an apoptosis of cells in several organs including the cerebrum and liver under the influence of hypercytokinemia is a possible mechanism of the disease.

Key words apoptosis, cytochrome c, hypercytokinemia, influenza-associated encephalopathy.

Frequently seen during the winter season are children suffering from influenza infection who exhibit complications including typical febrile convulsion, decreased sensorium, delirium, and finally deep, irreversible coma. Cases of such patients with encephalopathy are reported to be more frequent in East Asia than in the west.[1–3] In Japan, the total number of fatal cases of influenza encephalitis/encephalopathy in the 1997/1998 seasons was estimated at 100–200.[4] Togashi *et al.* reported that 64 infants and children developed encephalitis/encephalopathy during five influenza seasons in Hokkaido, the northern island of Japan.[5]

Although several types of influenza-associated encephalopathy have been reported clinically,[6] the pathological

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mechanism of influenza-associated encephalopathy still remains to be clarified. Current treatment of patients with the disease is restricted to early administration of viral inhibitors such as amantadine, zanavimir and oseltamivir and occasional plasma exchange and/or mild hypothermia therapy.[7] This is because the evaluation of effectiveness of these therapies is difficult and no appropriate marker has been found to monitor the severity or the progression of the disease.

With this in mind, we compared several proinflammatory cytokines, acute phase proteins, endothelial markers and also cytochrome c to find out pathogenic and clinical markers. Although cytochrome c is an intramitochondrial protein in intermembrane spaces and is involved in the electron transport system for oxidative phosphorylation, it also triggers the execution of apoptosis. In our retrospective study, we found that the serum level of cytochrome c in addition to tumor necrosis factor (TNF)-α and interleukin (IL)-8 is apparently elevated in patients with influenza-associated encephalopathy as compared with those without it. The elevation of serum cytochrome c reflects the severity of the disease and, furthermore, suggests that apoptosis of tissues including those of the cerebrum occurs in patients with influenza-associated encephalopathy under the influence of hypercytokinemia.

Materials and methods

Patients

All 53 patients involved in this study were diagnosed with influenza virus infection with influenza antigen detection kits (Directigen Flu A, Becton Dickinson, USA; FIU OIA, BIOSTAR, Australia; or Influ A and B-AD, Denka Seiken, Japan). Elevation of hemoaglutination inhibition antibody (HI-Ab) was also confirmed in some patients.

In this study, we defined the patients as suffering from influenza-associated encephalopathy if they showed: (i) clinical symptoms and signs compatible with acute encephalitis/encephalopathy, and exclusion of bacteria or fungal infection and all other neurological, vascular, metabolic, endocrine, toxic, and drug-induced disorders; (ii) isolation of influenza virus from the throat, or a fourfold increase in the antibody titer determined by means of the hemagglutination inhibition test and/or virus antigen detection in the throat with Kits; and (iii) the presence of cerebral edema, bleeding or acute necrotizing encephalopathy (ANE) confirmed by computed tomography (CT) or magnetic resonance imaging (MRI).

The profiles of 11 patients with influenza-associated encephalopathy are listed in Table 1. Most patients with encephalopathy showed multiorgan dysfunction to varying degrees. Patients 2, 3, 6, 7 and 11 suffered from hepatic and other organ dysfunction in which aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also elevated. Renal dysfunction was found in patients 1, 3, 4, 5, 6, 7 and 8. Thrombocytopenia was found in patients 1, 2, 3, 4, 6, 7 and 10. Rhabdomyolysis was found in patient 7. The sera of seven patients (1, 2, 3, 4, 5, 10 and 11) were sequentially stocked for more precise analysis.

All the 42 remaining patients with influenza infection were admitted for several days at hospitals in Kumamoto city with symptoms of high-grade fever and/or febrile convulsion. They were 20 boys and 22 girls and were 2 years 6 months ± 2 years 5 months old. They had no apparent biochemical abnormalities and no residual neurological sequelae on discharge.

All the sera were stored at –20°C on admission and subsequently with parental consent.

Excluded patients

In this study, we excluded four deceased patients (a 1-year-old female, a 1-year and 3-month-old male, a 2-year-old female and a 3-year-old male), even though they were suspected to have had an influenza-associated encephalopathy, because they were dead on arrival and biochemical data were limited, without CT and MRI findings.

Methods

Cytokine levels in sera were detected by the the following enzyme-linked immunosorbent assay (ELISA) kits: soluble E-selectin, sE-selectin ELISA ver2 (Bender med System); sTM, TM test (Teijin; Teijin Diagnostics); TNF-α, Human TNF-α Cytoscreen Immunoassay Kit (Bioscorce International); sFas, sFas ELISA Kit (Medical and Biological); sFas-L, sFas Ligand ELISA Kit (MBL), and IL-6 and IL-8 (Biotrak) IL6 and IL-8 human ELISA system (Amersham Pharmacia, NJ, USA). A normal range of these markers is shown in Table 2, and the values were reconfirmed by normal volunteers.

For serum cytochrome c assay, we developed a sandwich enzyme immunoassay (EIA) system.[8] A total of 50 μL of serum samples were placed in a polystyrene plate that had been coated with mouse antihuman cytochrome c monoclonal antibody (Fujisawa pharmaceutical Co. Ltd, Osaka, Japan). After 1 h incubation at room temperature, the plate was washed with buffer and incubated further for 1 h with rabbit anticytochrome c antibody IgG Fab fragment conjugated with horseradish peroxidase. Reaction buffer containing 2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid and H_2O_2 was added to all wells and incubated at room temperature for 1 h. The reaction was terminated with NaN_3 and absorbance at 405 nm of the reduced products was determined. The serum cytochrome c level of non-infected controls was

Table 1 Profile of the patients with influenza-associated encephalopathy

	Age	Sex	Onset days	Detection Kits	HI-Ab	JCS	CT/MRI	Outcome	Cyto c ng/mL	AST IU/L	Cr mg/dL	Plat 10^4/mL	CK U/L
1	3 years 2 months	M	1	FIU OIA		> 200	Diffuse	Died	50	53	1.2	2.9	–
2	2 years	M	1	Influ B		100–200	Diffuse	CNS sequela	56	6830/9520	ND	4.9	361/2250
3	2 years	F	1	Influ B		100	Diffuse	Improved	46	13 884/14 909	1.4	4.9	580
4	1 year 8 months	F	2		H3N2	300	Diffuse	Died	35	126	1.4	+	–/516
5	4 years	M	17 h	PCR/Influ A		300	ANE	Improved	32	102/695	1	–	–
6	3 years 2 months	F	1	PCR/Influ A	H3N2	30–300	Diffuse	Died	30.5	53 340	Renal failure	1.8	1139
7	6 years	M	0	Influ A	H3N2	3–200	Diffuse	Died	24	1174/6168	1.4	10.7	124 600
8	7 years	M	1	Influ A	H3N2	300	Lobar	CNS sequela	11	37	1.3	18.3	89
9	9 years	M	1	Influ A	H1N1	300	Lobar	CNS sequela	5.8	28/48	0.5	–	n.d.
10	1 month	M	0		H3N2	300	Mild edema	CNS sequela	2.4	51/33	0.3	+	n.d.
11	2 years	F	7 h	Influ A		300	ANE	Improved	1.2	102/695	0.7	–	n.d.

'Onset' means time between appearance of symptom and admission. FLU OIA and Influ A or B are influenza virus detection kits. Biochemical data are shown as level on admission/maximum during the course.

ANE, acute necrotizing encephalopathy; AST, aspartate aminotransferase (IU/L); CK, creatinine phosphokinase (U/L); CNS, central nervous system; Cr, creatinine (mg/dL); Cyto c, cytochrome c (ng/mL); F, female; HI-ab, hemoagglutination-antibody; H3N2 and H1N1, type of influenza viruses; JCS, Japanese coma score; M, male; –, normal; ND, not done; Plat, platelet (+ or –, with or without thrombocytopenia; X 104/μL).

less than 0.05 ng/mL. That of patients suffering from influenza infection was stable (0.1 ± 0.2 ng/mL). The level of serum cytochrome c was reproducible even after several freezing and thawing procedures.

Statistical procedures

The comparisons between the groups were performed by the Mann–Whitney rank sum test. The 0.05 level of probability was used as a significance criterion.

Results

We measured the serum levels of proinflammatory and acute phase proteins from 11 patients with influenza-associated encephalopathy and 42 other influenza patients. The statistical evaluation of serum concentrations of soluble E-selectin, sTM, TNF-α, sFas, sFas-ligand, IL-6, IL-8, and cytochrome c in the patients with and without influenza-associated encephalopathy is shown in Table 2. It is apparent that the level of cytochrome c was markedly elevated in the patients with influenza-associated encephalopathy as compared with the normal range and that of other influenza patients. The levels of TNF-α and IL-8 were also significantly elevated in the patients with influenza-associated encephalopathy. The *P*-values for these are, however, less significant, indicating that elevation of cytochrome c is the most reliable marker among the serum proteins tested for estimating the severity of influenza-associated encephalopathy.

As shown in Table 1, the level of serum cytochrome c in patients with influenza-associated encephalopathy correlated well with their clinical outcomes. Among the seven patients whose serum contained more than 20 ng/mL cytochrome c on admission, four died and one had severe central nervous system sequelae. Only two patients who received high dose gamma globulin therapy (patient 5) and/or cyclosporinee therapy (patient 3) survived. The concentration of cytochrome c in sera from the surviving patients decreased during treatment. Three patients (8, 9 and 10) with a greater than 2 ng/mL cytochrome c concentration developed severe neurological sequelae.

Cytochrome c levels of several patients who were subsequently analyzed are shown in Table 3. It appears that levels remained high in patients 1 and 4, who died later, but decreased after admission in patients 2, 3 and 5 who subsequently survived. In patient 10 who showed CNS sequelae, the level was slightly high on admission, but increased to 14 ng/mL on the third day of admission. Those of patient 11 remained at low levels as shown in Table 3. In patient 1, serum cytochrome c levels were assayed twice a day (with permission), they stayed high for the first 2 days and then decreased for 3 days and rose again after the sixth day of admission.

Table 2 Statistical analysis of various markers in sera from patients with and without influenza-associated encephalopathy

Markers	Normal	without IE	with IE	*P*-value
Cytochrome c	< 0.05 ng/mL	0.3 ± 0.7 (42)	26.8 ± 19.5 (11)	< 0.001
TNF-α	0 pg/mL	0.9 ± 3.7 (17)	11.6 ± 17.1 (5)	0.003
IL-8	1.2–16.7 pg/mL	17.3 ± 16.1 (16)	339.8 ± 400.2 (8)	0.016
IL-6	0–149 pg/mL	49.8 ± 156.7 (17)	55.5 ± 125.7 (6)	0.35
Soluble TM	11–20 ng/mL	1.4 ± 0.8 (27)	17.4 ± 19.8 (6)	0.39
Soluble Fas	1–3.9 ng/mL	1.4 ± 0.8 (29)	1.7 ± 0.5 (6)	0.52
Soluble E-selectin	20–60 ng/mL	76.6 ± 43.7 (42)	133.7 ± 0.5 (6)	0.99

The Mann-whitney *U*-test was used to determine significant differences in each marker between the patients with or without encephalopathy ($P < 0.05$).

IE, influenza-associated encephalopathy; IL, interleukin; Patients, number of patients examined; TNF-α, tumor necrosis factor-α.

Table 3 Time course of cytochrome c concentration after admission of patients who were analyzed over time. Serum cytochrome c levels were assayed twice a day (morning and evening) with permission in the case of patient 1

Patients	Days after admission									
	1	2	3	4	5	6	7	8	9	< 10
1	50/68	62/80	9.4/10.5	5.5/9.8	13/14.5	96	110/90	96		
2	56				6	5.8		5.3		3.3
3	46	37							6.2	8.1
4	35							98		66
5	32		0				0			0
10	2.4		14				9		3	
11	1.2	2.7	0	0			0			0

Discussion

We showed in this paper that serum concentration of cytochrome c was extremely high in the patients with influenza-associated encephalopathy. The serum levels of cytochrome c well reflect the severity of the disease and clinical outcome of the patients. The four excluded patients who were dead on arrival after influenza infection also showed high levels of serum cytochrome c (75, 58, 42 and 28.5 ng/mL). All patients had more than 2 ng/mL cytochrome c on admission or subsequently had severe sequelae, except the two who had received high dose gamma globulin therapy (patient 5) and/or cyclosporinee therapy (patient 3) for alleviation of hypercytokinemia. In contrast, two out of the 42 patients without influenza-associated encephalopathy who had more than 2 ng/mL of cytochrome c in the serum on admission developed persistent convulsions a few months later.

In patients 2, 3, 6 and 7, but not in the others, AST, ALT, and creatinine phosphokinase (CK) were elevated as well as cytochrome c. The other markers (creatinine, platelets, creatine phosphokinase etc.) were also elevated in some but not others, as in Table 1. From the view points of cytokine and biochemical markers, cytochrome c appeared to be a good marker for evaluating the clinical severity of influenza-associated encephalopathy.

At present, the following evidence has been reported about the relation between apoptosis and influenza virus infection: (i) an increase of mitochondrial permeability was reported in Reye's syndrome[9] which was supposed to be associated with influenza infection and aspirin side-effects; (ii) during apoptosis of the cell, cytochrome c triggers the execution phase of apoptosis by massive translocation into the cytoplasm;[10] (iii) in our study, serum cytochrome c on admission was elevated in the patients with but not without influenza-associated encephalopathy; (iv) in the biopsy specimens from patients 2 and 3, DNA breakage cells were detected at the early stage of hepatic failure by the TUNEL staining method (DeadEnd Colorimetric TUNEL System, Promega, Tokyo, Japan); and (v) it had already been confirmed that serum cytochrome c levels increased in MODS to MOD,[8] but did not increase in patients with other viral infections such as, acute hepatitis, upper respiratory viral infections, and enterovirus 71-rhomb encephalitis (H. Nunoi, unpubl. obs., 1998). Based on the evidence reported and that obtained in the present investigation, we believe that apoptosis is induced in several tissues of patients with influenza-associated encephalopathy, resulting in the release of cytochrome c from mitochondria.

We speculate that some cytokines may have induced apoptosis of the cells and organs in these patients. As serum TNF-α and IL-8 were significantly elevated as shown in

Table 2, it is possible that TNF-α is one of the inducers of apoptosis in this disease because TNF-α plays an important role in the apoptosis of influenza-infected cells *in vitro*[11] and serum TNF-α shows considerable correlation with the severity of the disease. In addition, pharmacokinetic studies have revealed that TNF-α has a rapid clearance rate with a half-life of 15–30 min,[12] so that the levels were supposed to be influenced by the stage of the disease. Although it was proposed that IL-6 had a predictive value for patients with influenza-associated encephalopathy,[6,13] we did not observe a significant difference in the levels of IL-6 between those with and without the disease ($P = 0.35$). It is still uncertain which cytokine is causative and pathogenic in the disease and how they are induced by influenza virus infection.[13–16]

Of certainty, is that the present results indicate that the serum level of cytochrome c is a reliable indicator for estimating the severity of influenza-associated encephalopathy. A follow-up survey of serum cytochrome c in patients with the disease is important for the prediction of their clinical outcome.

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References

1 Oseasohn R, Adelson L, Kaji M. Clinicopathologic study of thirty-three cases of Asia Influenza. *N. Engl. J. Med.* 1959; **260**: 509–18.
2 Yoshikawa H, Yamazaki S, Watanabe T *et al.* Study of influenza-associated encephalitis/encephalopathy in children during the 1997 to 2001 influenza seasons. *J. Child Neurol.* 2001; **16**: 885–90.
3 Okabe N, Yamashita K, Taniguchi K *et al.* Influenza surveillance system of Japan and acute encephalitis and encephalopathy in the influenza season. *Pediatr. Int.* 2000; **42**: 187–91.
4 Reichert TA, Sugaya N, Fedson DS, Glezen WP, Simonsen L, Tashiro M. The Japanese experience with vaccinating schoolchildren against influenza. *N. Engl. J. Med.* 2001; **344**: 889–96.
5 Togashi T, Matsuzono Y, Narita M. Epidemiology of influenza-associated encephalitis-encephalopathy in Hokkaido, the northernmost island of Japan. *Pediatr. Int.* 2000; **42**: 192–6.
6 Mizuguchi M, Abe J, Mikkaichi K *et al.* Acute necrotizing encephalopathy of childhood: a new syndrome presenting with multifocal symmetric brain lesion. *J. Neurol. Neurosurg. Psychiatry* 1995; **58**: 551–61.
7 Munakata M, Kato R, Yokoyama H *et al.* Combined therapy with hypothermia and anticytokine agents in influenza A encephalopathy. *Brain Dev.* 2000; **22**: 373–7.
8 Adachi N, Hirota M, Hamaguchi M *et al.* Serum cytochrome c level as a prognostic indicator in patients with systemic inflammatory response syndrome. *Clin. Chim. Acta* 2004; **342**: 127–36.
9 Trost LC, Lemasters JJ. The mitochondrial permeability transition: a new pathophysiological mechanism for Reye's syndrome and toxic injury. *J. Pharmacol. Exp. Ther.* 1996; **278**: 1000–5.
10 Liu X, Kim CN, Yang J *et al.* Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* 1996; **86**: 147–57.
11 Takizawa T, Tatematsu C, Ohashi K *et al.* Recruitment of apoptotic cysteine proteases (caspases) in influenza virus-induced cell death. *Microbiol. Immunol.* 1999; **43**: 245–52.
12 Harris SG, Smith RS, Phipps RP. 15-deoxy-Delta 12,14-PGJ2 induces IL-8 production in human T cells by a mitogen-activated protein kinase pathway. *J. Immunol.* 2002; **168**: 1372–9.
13 Aiba H, Mochizuki M, Kimura M *et al.* Predictive value of serum interleukin-6 level in influenza virus-associated encephalopathy. *Neurology* 2001; **57**: 295–9.
14 Ito Y, Ichiyama T, Kimura H *et al.* Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy. *J. Med. Virol.* 1999; **58**: 420–5.
15 Hinshaw VS, Olsen CW, Dybdahl-Sissoko N *et al.* Apoptosis: a mechanism of cell killing by influenza A and B viruses. *J. Virol.* 1994; **68**: 3667–73.
16 Ichiyama T, Isumi H, Ozawa H *et al.* Cerebrospinal fluid and serum levels of cytokines and soluble tumor necrosis factor receptor in influenza virus-associated encephalopathy. *Scand. J. Infect. Dis.* 2003; **35**: 59–61.

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CC chemokine receptor 4 ligand production by bronchoalveolar lavage fluid cells in cigarette-smoke-associated acute eosinophilic pneumonia

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Abstract

We examined the production of macrophage-derived chemokine (MDC/CCL22) and thymus- and activation-regulated chemokine (TARC/CCL17) by bronchoalveolar lavage fluid (BALF) cells in cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP). The CC Chemokine Receptor 4 (CCR4) ligand levels in BALF from patients with CS-AEP were considerably higher than those in healthy volunteers and correlated well with Th2 cytokine levels. Interleukin-4 enhanced CCR4 ligand production. MDC expression was observed in CD68-positive cells from patients with CS-AEP and in healthy control smokers. In contrast, TARC expression in CD68- or CD1a-positive cells was detected only in CS-AEP. An in vivo cigarette smoke challenge test induced increases in CCR4 ligands in the BALF and in the cultured supernatant of BALF adherent cells. These results suggest that alveolar macrophages and dendritic cells contribute to the pathogenesis of CS-AEP by generating CCR4 ligands, probably in response to cigarette smoke.

Keywords: Macrophage-derived chemokine; Thymus- and activation-regulated chemokine; Cell culture; Cigarette-smoke-associated acute eosinophilic pneumonia; Immunocytochemistry

Introduction

Acute eosinophilic pneumonia (AEP) is characterized by an acute febrile illness with severe hypoxemia, diffuse pulmonary infiltrates, and an increase in bronchoalveolar lavage fluid (BALF) eosinophils [1]. It is a reversible cause of noninfectious respiratory failure. Several etiologic factors, including drugs [2,3], environmental agents [4–6], and cigarette smoke [7,8], are suspected in the pathogenesis of AEP. In particular, cases of cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP) are accumulating based on positive cigarette smoke challenge tests [7–11]. In the pathophysiology of AEP, T helper 2 (Th2) cells are likely to have a significant role in promoting eosinophilic inflammation in this disorder [12–14]. Indeed, in CS-AEP, the levels of Th2 cell-derived cytokines, such as interleukin (IL)-4 and IL-5, are increased in BALF after exposure to cigarette smoke [9,15]. CC chemokine receptor 4 (CCR4) is preferentially expressed on the surface of Th2 cells [16]. In this context, we demonstrated that BALF from patients with eosinophilic pneumonia contains elevated concentrations of thymus- and activation-regulated chemokine (TARC/CCL17) [13], a CCR4 ligand. The cell sources as well as the regulatory mechanisms of TARC production, however, remain uncertain. In addition, whether cigarette smoke induces TARC production in patients with CS-AEP is of great interest.

Macrophage-derived chemokine (MDC/CCL22), another CCR4 ligand, is considered crucial for the pathogenesis of various allergic disorders [17–19]. Although both MDC and TARC signal through CCR4 as their receptor and both can

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act as Th2-type T cell chemoattractants, there are some differences between these chemokines in the pathogenesis of allergic disorders. For example, TARC is significantly increased in asthmatic sera, but MDC is not [20]. Similarly, stimulation with the same proinflammatory cytokines produces TARC, but not MDC, in bronchial epithelial cells [21]. Moreover, MDC is the sole intestinal epithelial-derived factor responsible for recruiting CCR4-positive T cells [22]. Furthermore, in NC/Nga mice exhibiting atopic dermatitis-like lesions, both MDC and TARC are involved in the pathogenesis; however, there are distinct cell types producing each chemokine. Briefly, dermal dendritic cells are the main source of MDC, whereas keratinocytes are the main source of TARC [23]. Therefore, expression and production of these CCR4 ligands should be comparatively examined in the pathogenesis of CS-AEP.

In this report, we measured MDC and TARC concentrations in BALF from patients with CS-AEP and in healthy volunteers, and tried to determine the cell sources of MDC and TARC. Moreover, to examine the regulation of these chemokines, we cultured BALF cells under stimulation with several cytokines or with cigarette smoke.

Methods

Patient recruitment and bronchoalveolar lavage

AEP was diagnosed according to the original criteria with some modifications as follows: acute febrile illness, severe hypoxemia, diffuse pulmonary infiltrates, increased percentage of eosinophils in BALF, and no pulmonary or systemic infection [1]. The cause of the patients' disease was suspected to be exposure to cigarette smoke; alternative causes such as drugs [2,3] and environmental agents [4–6] were ruled out. Three patients had a history of bronchial asthma and another patient had a history of both bronchial asthma and atopic dermatitis. Bronchoalveolar lavage (BAL) was performed principally to diagnose each disease after obtaining informed consent. Written informed consent was provided by the healthy volunteers. The healthy control subjects consisted of those who were free from any clinical signs of lung disease at the time of the BAL procedure and had no history of lung disease. There were nine current smokers and seven non-smokers. The nine healthy volunteers who underwent BALF cell culture consisted six current smokers and three non-smokers. The BAL procedure and cell analysis was performed as described previously [13]. BAL was performed by injection of 150 ml of saline into the middle lobe or left lingula. The cells were counted in a hemocytometer. A 100-μl aliquot of cells (1×10^6/ml) was subjected to cytocentrifugation (Cytospin, Shandon Southern, Runcorn, UK) and air-dried to obtain differential cell counts after staining with May–Grünwald–Giemsa stain. To determine the phenotype of the lymphocytes in the BALF, a fluorescence-activated cell sorter scan was performed by the direct immunofluorescence method using anti-CD2, anti-CD4, and anti-CD8 monoclonal antibodies (MoAb). The cell-free supernatants were stored at −80°C until further analysis.

Measurement of chemokines and cytokines in the BALF and culture supernatant

Chemokine and cytokine concentrations in the BALF and culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) according to each manufacturer's protocol. The minimum detectable doses in the ELISA system were MDC, 62.5 pg/ml; TARC (Genzyme, Minneapolis, MN), 7 pg/ml; IL-4, 0.27 pg/ml; IL-5, 4 pg/ml; IL-13 (Biosource Inc., Camarillo, CA), 12 pg/ml; and granulocyte-macrophage colony stimulating factor (GM-CSF; R&D Systems, Adbington, UK), 3 pg/ml.

Cell culture

BALF cells collected by centrifugation were suspended in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% fetal calf serum, and cultured in cell culture dishes for 30 min at 37°C in a 5% CO_2 incubator. After non-adherent cells were removed by washing the dishes with sterile saline three times, the remaining adherent cells were removed and collected. The final concentration of adherence-purified viable BALF cells was adjusted to 0.5×10^5/ml. These cells were cultured in the above medium for 48 h at 37°C in a 5% CO_2 incubator. The cells were cultured with various stimuli; 10 ng/ml lipopolysaccharides (Sigma-Aldrich Chemical Co., St Louis, MO), 1000 U/ml tumor necrosis factor-α (TNF-α; Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan), 20 ng/ml IL-4 (R&D Systems), and 500 U/ml interferon-gamma (IFN-γ; Shionogi, Osaka, Japan). The culture supernatants were stored at −80°C until further analysis.

Antibodies

The antibodies used for immunocytochemistry and their corresponding dilutions or concentrations were as follows: rabbit anti-human MDC polyclonal antibody (PoAb) (PeproTech EC, London, UK) at 0.4 μg/ml; goat anti-human TARC PoAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 2 μg/ml; mouse anti-human CD68 MoAb (Dako, Kyoto, Japan) at 0.9 μg/ml as the cell marker for macrophages; mouse anti-human CD1a MoAb (Santa Cruz Biotechnology) at 2 μg/ml as the cell marker for dendritic cells; biotin-labeled goat anti-rabbit IgG (H + L), biotin-labeled horse anti-goat IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA), alkaline phosphatase (AP)-labeled goat anti-mouse IgG (H + L), AP-labeled horse anti-mouse IgG (H + L) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) at 1:200. As negative controls, the same

species and isotype-matched immunoglobulins were used; goat IgG, rabbit IgG, mouse IgG1 (DAKO).

Immunocytochemistry

Aliquots of BALF cells (1×10^6 cells) were pelleted onto glass slides and fixed in cold acetone for 10 min. After incubation in a solution containing 1% bovine serum albumin and 10% normal goat serum or normal horse serum, the slides were incubated with rabbit anti-human MDC PoAb or goat anti-human TARC PoAb at room temperature for 2 h. Negative controls were run by replacing the primary antibody with rabbit IgG or goat IgG at the concentration of each primary antibody. After blocking endogenous peroxidase activity by incubation in 0.3% H_2O_2 and 0.1% NaN_3 for 30 min, the bound antibodies were labeled with biotin-labeled goat anti-rabbit IgG (H + L) or biotin-labeled horse anti-goat IgG (H + L). The avidin–biotin–peroxidase complex (ABC) solution (Vector Laboratories) was then applied sequentially for 30 min. The peroxidase activity was visualized with amino-3, 9-ethylcarbazole (AEC); the working AEC solution was applied and incubated for 10 min. The sections were counterstained with Mayer's hematoxylin and mounted with Geltol (Lipshaw Immunon, Pittsburgh, PA).

Double staining

We also performed immunocytochemical double staining using a combination of the ABC method and the AP technique. The slides were incubated at 4°C overnight with rabbit anti-human MDC PoAb or goat anti-human TARC PoAb. After blocking endogenous peroxidase activity in 0.3% H_2O_2 and 0.1% NaN_3 for 30 min, the slides were incubated for 30 min with biotin-labeled goat anti-rabbit IgG or biotin-labeled horse anti-goat IgG and then ABC solution was applied sequentially for 10 min. The peroxidase activity was visualized with AEC. Next, the slides were incubated with a second primary antibody, mouse anti-human CD68 MoAb, or mouse anti-human CD1a MoAb followed by AP-labeled goat anti-mouse IgG or AP-labeled horse anti-mouse IgG. AP activity was the represented with the Vectastain ABC-AP substrate kit III (Vector Laboratories). Endogenous AP activity was inhibited by the addition of 1 mM levamisole to the reaction mixture. Negative controls were run by replacing primary antibodies with an irrelevant isotype-matched immunoglobulin. Cross-reactivity of anti-MDC or TARC antibody with the second step primary antibody was examined processing four pairs, i.e., anti-MDC or TARC antibody (+)/second-step primary antibody (+), (+)/(−), (−)/(+), and (−)/(−).

Cigarette smoking challenge test

The cigarette smoking challenge test was approved by the ethics committee of Oita University Faculty of Medicine. Of 10 patients with CS-AEP, 6 were teenagers and were thus excluded from the challenge test because cigarette smoking is forbidden for teenagers by Japanese law. Finally, we obtained informed consent for the challenge test from one of four CS-AEP patients who were over 20 years of age. The other three patients refused to undergo the challenge test.

Statistical analysis

Results are presented as median values, with minimum and maximum values as the range. The Kruskal–Wallis test was used to compare values of the different groups. In case of a significant difference between groups, intergroup comparisons were assessed by a nonparametric method using the Mann–Whitney U test. Correlation coefficients were determined using the Pearson's linear regression analysis. A P value of $P < 0.05$ was considered significant.

Results

Patient characteristics and BALF findings

Ten patients with CS-AEP were enrolled in the study. Detailed characteristics of these patients are described in Table 1. The median age of the CS-AEP group was 18 years and 6 patients were teenagers. The control group consisted of nine smoking-healthy volunteers (s-HV) and seven non-smoking-healthy volunteers (n-HV), each group with a median age of 25 years. Although the CS-AEP group consisted of four patients with atopic history and the other two groups had no atopic history, there were no significant differences between the three groups ($P = 0.0557$). Nine patients had started smoking within 1 month before the onset of symptoms and met our criteria of CS-AEP. The remaining patient was exclusively exposed to cigarette smoke passively for a few months before the onset of the symptoms and was diagnosed with CS-AEP. Most patients with CS-AEP spontaneously improved after smoking cessation without corticosteroid therapy. One patient with CS-AEP was receiving corticosteroid therapy prior to undergoing BAL. The cell concentration in BALF from patients with CS-AEP was significantly higher than that of healthy volunteers. Patients with CS-AEP had significantly increased percentages of lymphocytes and eosinophils in the BALF than did the healthy volunteers.

MDC and TARC concentrations are elevated in BALF from patients with CS-AEP

The MDC and TARC concentrations in BALF from patients with CS-AEP and healthy volunteers are shown in Fig. 1. The MDC concentrations in the CS-AEP group (median, 1518 pg/ml; range, 0–15,955 pg/ml) were considerably higher than those of each healthy volunteer group,

Table 1
Characteristics of study population

Characteristics	CS-AEP (n = 10)	s-HV (n = 9)	n-HV (n = 7)	P value
Age (yr), median (min, max)	18 (15, 21)	25 (21, 32)	25 (21, 32)	$P < 0.005$
Male (%)	80	89	86	N.S.
Smoking status				
Current smokers (%)	90	100		N.S.
Number/day, median (min, max)	8 (3, 40)	20 (8, 20)		N.S.
Smoking more than a month (%)	0	100		$P < 0.0001$
BALF analysis				
TCC ($\times 10^5$ cells/ml), median (min, max)	7 (1, 87)	2 (0.9, 3.9)	1.8 (1.2, 2)	$P < 0.005$
Lym (%), median (min, max)	11 (6, 44)	2.3 (1.7, 7)	6.2 (1, 7)	$P < 0.001$
Eo (%), median (min, max)	50 (26, 83)	0 (0, 0.7)	0 (0, 0.7)	$P < 0.001$
CD4/CD8 ratio, median (min, max)	1.6 (1, 4.9)	1.7 (0.2, 13)	1.6 (0.9, 3.1)	N.S.

Definition of abbreviations: CS-AEP = cigarette-smoke-associated acute eosinophilic pneumonia (n = 10), s-HV = smoking-healthy volunteer (n = 9), n-HV = non-smoking-healthy volunteer (n =7), yr = years, BALF = bronchoalveolar lavage fluid, TCC = total cell concentration, Lym = lymphocyte, Eo = eosinophil, min = minimum, max = maximum, N.S. = not significant. Comparison between groups were assessed by Kruskal–Wallis test.

and the differences were statistically significant (Fig. 1A). The median TARC concentration in the BALF from patients with CS-AEP was 3536 pg/ml (range, 29–23,225 pg/ml), which was higher than that from each healthy volunteer group (Fig. 1B). There were two CS-AEP patients whose BALF CCR4 ligand levels were below 100 pg/ml (open

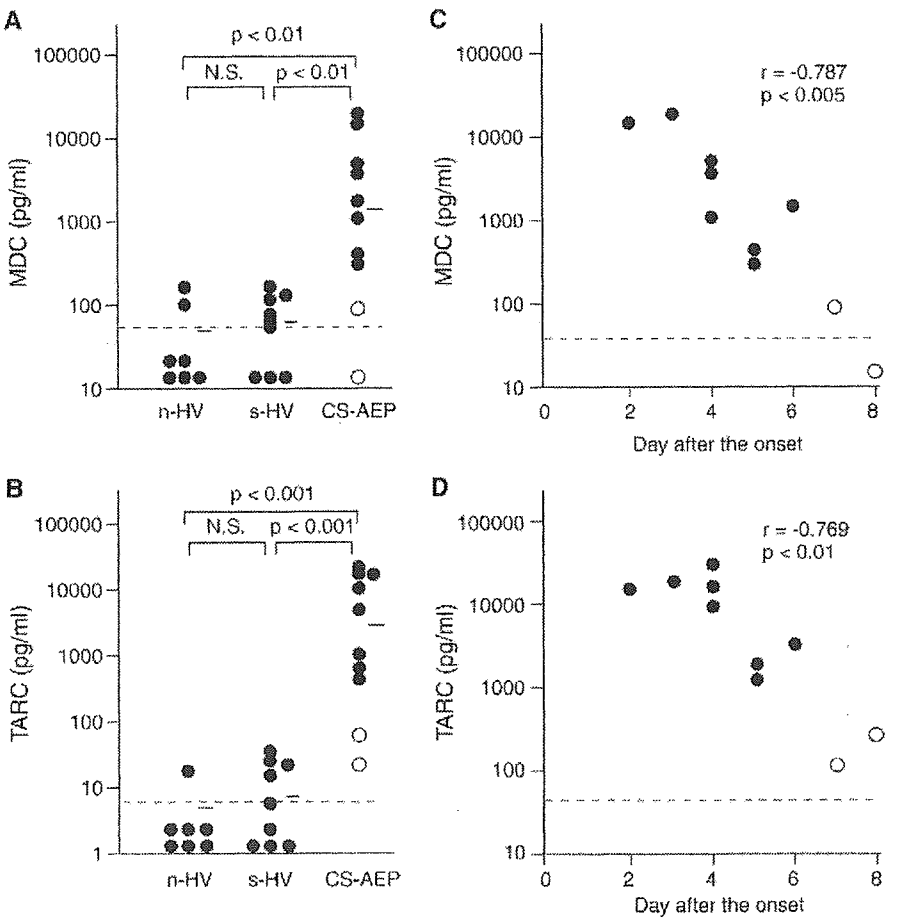

Fig. 1. (A, B) Concentrations of MDC and TARC in BALF obtained from non-smoking-healthy volunteers (n-HV; n = 7), smoking-healthy volunteers (s-HV; n = 9), and patients with cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP; n = 10). Each concentration was represented in the log scale. A short horizontal line represents the median value. The detectable limit of MDC and TARC are represented as a horizontal dotted line. For CS-AEP patients, BAL procedure was done within 6 days (closed circle) or over 6 days (open circle) from the onset of the symptoms. Pared comparisons were evaluated by Mann–Whitney U test. N.S., not significant. (C, D) Relationship between BALF CCR4 ligand from patients with CS-AEP and days after onset.

circles). They were the only two patients for whom over 6 days has passed from the onset of the symptoms (Figs. 1A–D). When we divided the CS-AEP group into CS-AEP patients with or without atopic history, there was no significant difference in the BALF CCR4 ligand levels (MDC; $P > 0.9999$, TARC; $P = 0.8312$). Furthermore, there was a significant negative correlation between CCR4 ligand levels and days after onset (Figs. 1C, D). When we examined the healthy volunteers, there were no significant differences in the BALF CCR4 ligand levels between smokers and non-smokers.

Correlations between MDC, TARC, and Th2 cytokines

There were strong correlations between the levels of MDC and IL-4 ($r = 0.738$, $P < 0.05$) and IL-13 ($r = 0.750$, $P < 0.05$), as well as between the levels of TARC and IL-4 ($r = 0.802$, $P < 0.005$), IL-5 ($r = 0.964$, $P < 0.0001$), and IL-13 ($r = 0.908$, $P < 0.0001$) in the BALF from patients with CS-AEP. There was a significant strong correlation between MDC and TARC levels in the BALF from patients with CS-AEP ($r = 0.731$, $P < 0.0001$).

IL-4 and GM-CSF concentrations were elevated in the BALF from patients with CS-AEP

We compared IL-4 and GM-CSF levels in the BALF between patients with CS-AEP and healthy volunteers. Concentrations of GM-CSF in the BALF from patients with CS-AEP were significantly higher when compared with s-HV or n-HV (Fig. 2B). BALF IL-4 was detected in 5 of 10 patients with CS-AEP, and in one of healthy volunteers. BALF IL-4 levels from patients with CS-AEP were significantly higher than those from s-HV or n-HV (Fig. 2A). When we divided CS-AEP patients into those with and without atopic history, there was no significant difference in the concentrations of IL-4 ($P = 0.3374$) or GM-CSF ($P = 0.2864$).

Production of MDC and TARC by BALF cells from healthy volunteers

Detectable levels of MDC were obtained from eight subjects, whereas only two subjects had detectable levels of TARC in the culture supernatant of adherence-purified BALF cells without cytokine stimulation. Our result indicated that neither lipopolysaccharides nor TNF-α affected MDC and TARC production in adherence-purified BALF cells. In contrast, production of MDC and TARC was enhanced by the addition of IL-4 (Tables 2 and 3). The combination of TNF-α and IL-4 induced increased secretion of MDC and TARC when compared with TNF-α without IL-4. There was no difference in the MDC and TARC levels between stimulation with TNF-α/IL-4 and IL-4 alone. When IFN-γ was added to the culture medium, MDC and TARC production were slightly decreased, but not to statistically significant levels. When we divided healthy volunteers into s-HV and n-HV, there was no significant difference in the levels of CCR4 ligand productions without cytokine stimulation or with any cytokine stimulation.

Increased MDC and TARC production in a patient with CS-AEP after a cigarette smoking challenge test

We performed a cigarette smoking challenge test in a 21-year-old man. He had started smoking 12 days before the appearance of high fever, severe cough, and dyspnea. Bilateral alveolar infiltrates, BALF eosinophilia, and negative findings of infection confirmed the diagnosis of CS-AEP. His symptoms rapidly improved by quitting smoking

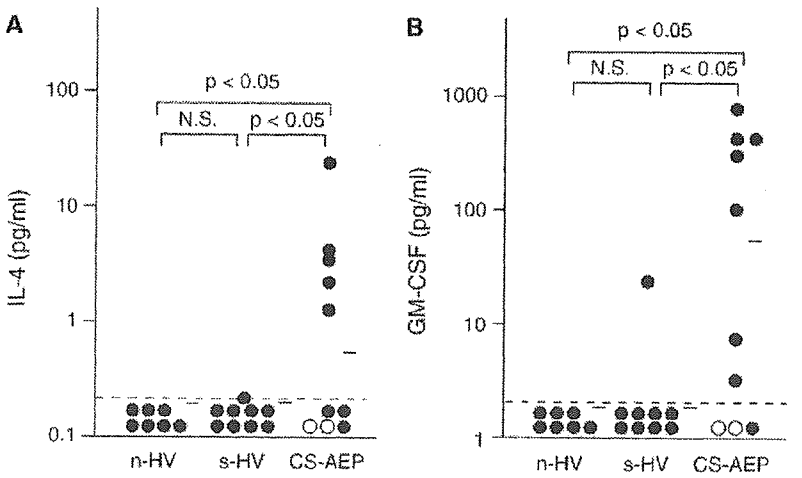

Fig. 2. Concentrations of IL-4 and GM-CSF in BALF from non-smoking-healthy volunteers (n-HV; $n = 7$), smoking-healthy volunteers (s-HV; $n = 9$), and patients with cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP; $n = 10$). Each concentration was represented in the log scale. A short horizontal line represents the median value. The detectable limit of IL-4 and GM-CSF are represented as a horizontal dotted line. For CS-AEP patients, BAL procedure was done within 6 days (closed circle) or over 6 days (open circle) from the onset of the symptoms. Paired comparisons were evaluated by Mann–Whitney U test. N.S., not significant.

Table 2
Production of MDC by adherence-purified BALF cells

Stimulus	MDC production (pg/ml)	
	HV (n = 9)	CS-AEP (n = 1)
Medium only	543 ± 181	4423
IL-4	2580 ± 632*	13,730
IFN-γ	183 ± 98	1515
LPS	456 ± 100	3795
TNF-α	499 ± 228	5121
TNF-α + IL-4	3826 ± 702**	5385
TNF-α + IFN-γ	220 ± 102	1980

They were presented as MDC concentrations (pg/ml) in the culture supernatant of adherence-purified BALF cells. Definition of abbreviations: HV = healthy volunteer (n = 9), CS-AEP = cigarette-smoke-associated acute eosinophilic pneumonia (n = 1). *P < 0.05 and **P < 0.01, compared with the non-stimulation group or the TNF-α stimulation group evaluated by Mann–Whitney U test. Values were represented as mean ± SEM.

without corticosteroid administration. Fifteen days after the symptoms disappeared, we administered the challenge test. Thirteen hours after the patient smoked three cigarettes in 45 min, he experienced high fever (38.3°C), general fatigue, and a nonproductive cough. Sixteen hours after exposure, BAL was performed when PaO_2 was 60.9 Torr.

The BALF obtained after the challenge revealed a markedly elevated eosinophil count (8.9×10^5/ml), compared with before the challenge (2.9×10^5/ml). The smoking challenge also induced an increase in the BALF lymphocyte population (before, 4.4×10^4/ml vs. after, 8.4×10^4/ml). This patient's chemokine levels in the supernatants of the purified cells harvested before the challenge with or without stimuli are shown in Tables 2 and 3. Although TARC concentrations in the culture supernatant before the challenge were similar to those of healthy volunteers, the MDC level before the challenge was already elevated compared with s-HV. MDC and TARC concentrations in the culture supernatant of adherence-purified BALF cells were highest in the presence of IL-4, whereas those induced by IFN-γ were lowest among the various cytokine stimuli. The concentrations of MDC and TARC in the BALF after the challenge were strikingly higher than those before the challenge (MDC, 84 pg/ml vs. 1286 pg/ml; TARC, 10 pg/ml vs. 2212 pg/ml) (Fig. 3). To determine whether BALF mononuclear cells secrete MDC and TARC in response to cigarette smoke, we cultured adherence-purified BALF cells. TARC levels in the culture supernatant after the challenge were 22 times higher than those observed before the challenge (41 pg/ml vs. 915 pg/ml) (Fig. 3B). In contrast, the MDC level was already high even before the challenge test and increased slightly from 4424 pg/ml to 6432 pg/ml (Fig. 3A) following the challenge.

Immunocytochemistry revealed MDC and TARC expression on BALF cells

Immunocytochemistry was performed on BALF cells from patients with CS-AEP and s-HV. Negative controls, which were run by replacing the primary antibodies with an irrelevant isotype-matched immunoglobulin, were uniformly non-reactive. MDC expression was observed in most large mononuclear cells obtained from patients with CS-AEP (Fig. 4B) as well as s-HV (Fig. 4A). Between patients with CS-AEP and controls, it was difficult to identify differences in the intensity and positive ratios in mononuclear cells. In contrast, there were no TARC-positive cells on slides from s-HV (Fig. 4C), while large mononuclear cells were faintly stained with anti-TARC MoAb in CS-AEP. More strikingly, TARC was very clearly detected on relatively small mononuclear cells from patients with CS-AEP (Fig. 4D), although the percentages were relatively low.

Determination of TARC-positive and MDC-positive cell phenotypes

To determine the phenotype of MDC- and TARC-positive BALF cells, double staining was performed for three patients with CS-AEP. CD68 and CD1a were used as cell markers for alveolar macrophages and dendritic cells, respectively [24–28]. Most large mononuclear cells were double-positive for MDC and CD68 (Fig. 4E) and were negative against anti-CD1a MoAb (Fig. 4F). Also, large mononuclear cells faintly stained with anti-TARC were positive for CD68. In contrast, a small number of TARC-strongly positive cells were CD1a-positive (Fig. 4H), but negative against anti-CD68 MoAb (Fig. 4G).

Discussion

We previously demonstrated that, in several diffuse lung diseases, BALF TARC levels are elevated in eosinophilic pneumonia [13]. Consistent with a recent report [29], there were high concentrations of another CCR4 ligand, MDC, present in the BALF of patients with CS-

Table 3
Production of TARC by adherence-purified BALF cells

Stimulus	TARC production (pg/ml)	
	HV (n = 9)	CS-AEP (n = 1)
Medium only	26 ± 16	41
IL-4	217 ± 113*	334
IFN-γ	6 ± 5	15
LPS	7 ± 5	23
TNF-α	11 ± 11	44
TNF-α + IL-4	432 ± 162**	133
TNF-α + IFN-γ	8 ± 8	16

They were presented as TARC concentrations (pg/ml) in the culture supernatant of adherence-purified BALF cells. Definition of abbreviations: HV = healthy volunteer (n = 9), CS-AEP = cigarette-smoke-associated acute eosinophilic pneumonia (n = 1). *P < 0.05 and **P < 0.01, compared with the non-stimulation group or the TNF-α stimulation group evaluated by Mann–Whitney U test. Values were represented as mean ± SEM.

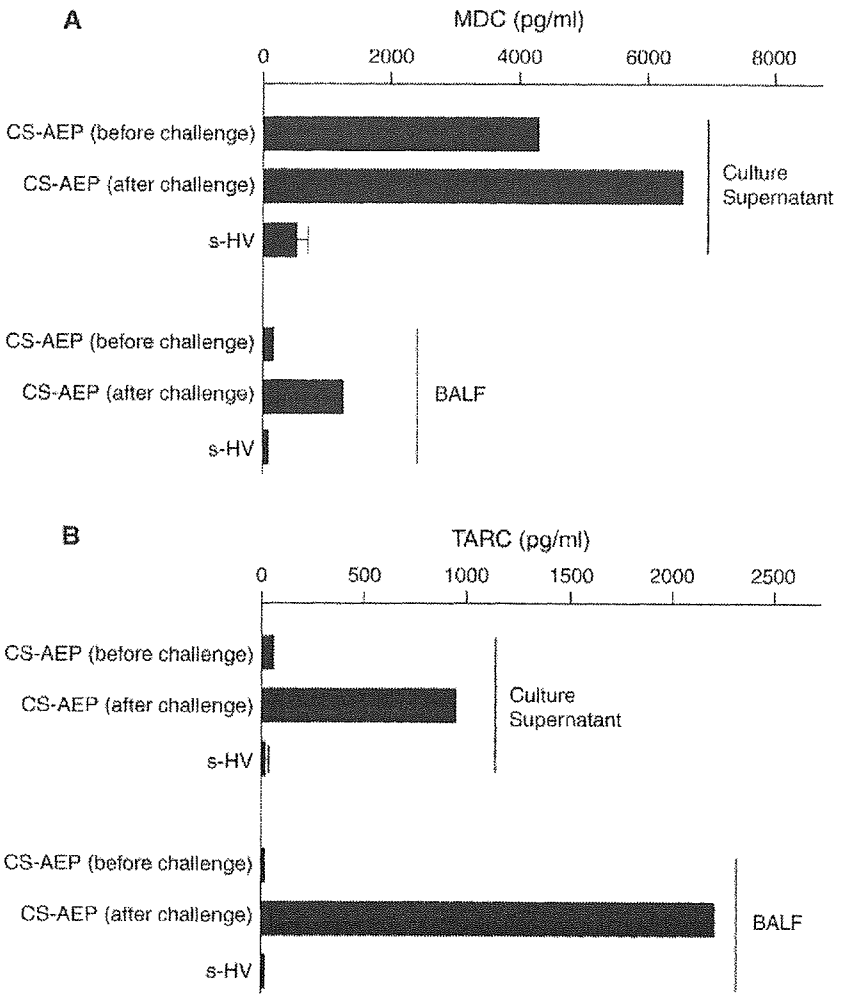

Fig. 3. Concentrations of MDC and TARC in the culture supernatants of adherence-purified BALF cells and BALF from a patient with cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP; $n = 1$) and smoking-healthy volunteers (s-HV; $n = 6$). MDC and TARC concentrations were compared from before cigarette smoking challenge test and 16 h after the challenge test.

AEP that were undetectable in most patients with hypersensitivity pneumonitis, sarcoidosis, or idiopathic pulmonary fibrosis (data not shown). In a murine model of asthma, MDC has a role in regulating the migration and accumulation of leukocytes within the tissue during the development of airway hyperreactivity [19]. Furthermore, high MDC production occurs in patients with atopic dermatitis and in NC/NgA mice exhibiting atopic dermatitis lesions [23]. Given the crucial role of MDC in allergic diseases, this CCR4 ligand as well as TARC might contribute to the pathogenesis of CS-AEP, probably by attracting CCR4-bearing Th2 cells from the peripheral circulation. Indeed, both MDC and TARC concentrations closely correlated with Th2-derived cytokines (e.g., IL-4, IL-5, and IL-13), with the exception of MDC and IL-5 levels in the BALF from patients with CS-AEP. Moreover, there are significant correlations between CCR4 ligand levels and the number of CCR4 + CD4 + T cells in the BALF of patients with eosinophilic pneumonia [29]. There are increased levels of soluble IL-2 receptor and increased numbers of activated T cells bearing HLA-DR antigen in BALF from patients with eosinophilic pneumonia [14]. Collectively, activated T cells might participate in the recruitment of eosinophils; however, it remains unclear whether CCR4 + CD4 + T cells were actually activated or producing Th2 cytokines in AEP, or whether there is an antigen to which they were responding. Further analysis is required to examine these possibilities. The time course of BALF MDC and TARC concentrations in our series revealed that the CCR4 ligand levels were extremely high within a couple of days from the onset of symptoms and then immediately declined over several days. This indicates that the function of MDC and TARC in CS-AEP is specific to the early phase of the disease. Thus, MDC and TARC might both participate in the pathogenesis of CS-AEP as well as other allergic disorders, but in atopic dermatitis and bronchial asthma, MDC and TARC originate from different cell sources [21–23].

When we examined the expression of CCR4 ligands in BALF cells, MDC was expressed in alveolar macrophages

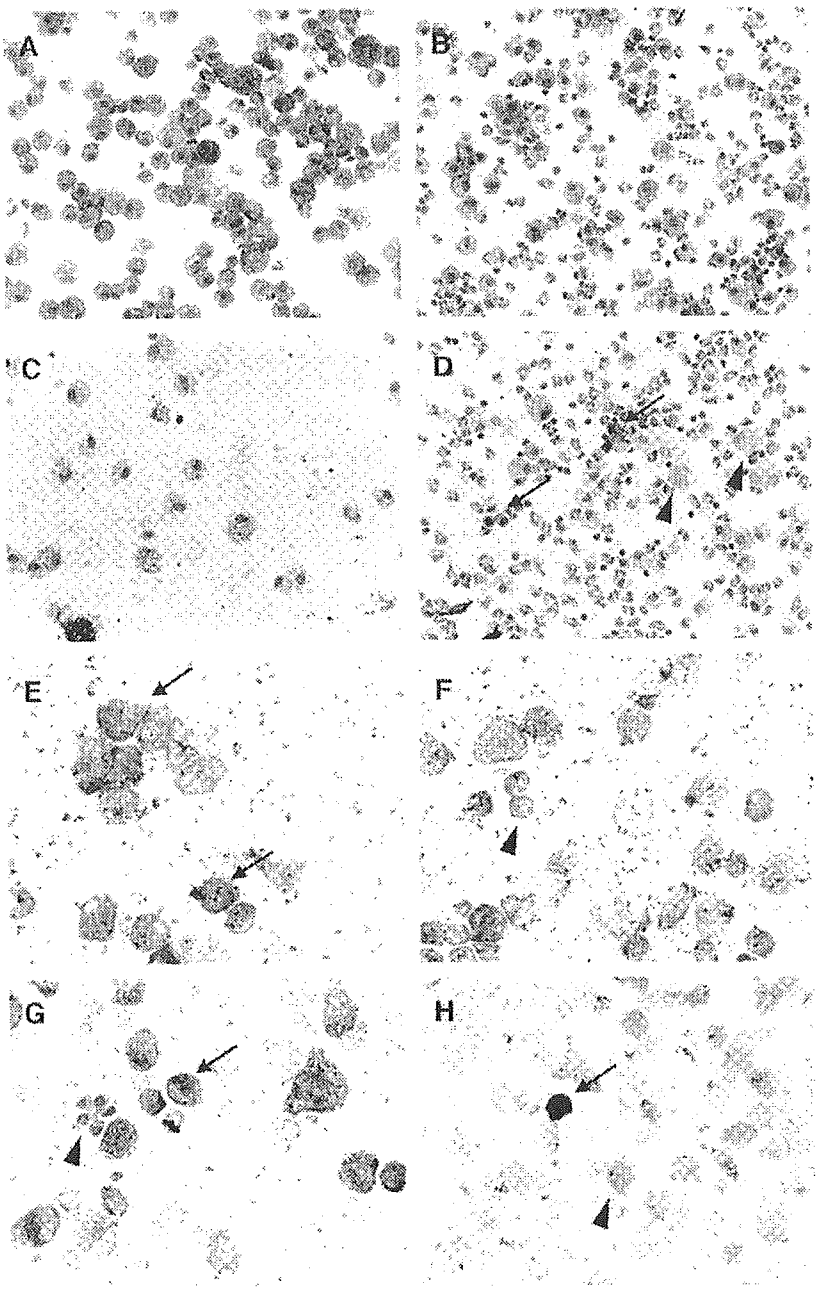

Fig. 4. Immunocytochemical staining of BALF cells with specific antibodies for MDC, TARC, CD68, and CD1a. (A) MDC expressions were observed in large mononuclear cells obtained from smoking-healthy volunteers (red). (B) MDC expressions were observed in large mononuclear cells obtained from cigarette-smoke-associated acute eosinophilic pneumonia (CS-AEP) patients (red). (C) TARC-positive cells were not observed on slides from any healthy volunteers. (D) Small mononuclear cells were strongly positive for TARC (red, arrow) and large mononuclear cells were faintly positive for TARC (red, arrowhead) on slides from CS-AEP patients. Double staining of BALF cells from a patient with CS-AEP (E–H). (E) Most large mononuclear cells were double-positive for MDC and CD68 (red + blue, arrow). (F) CD1a-positive cells were negative for MDC (blue, arrowhead). (G) Large mononuclear cells were double-positive for TARC and CD68 (red + blue, arrow), whereas TARC-positive small, round mononuclear cells were negative for CD68 (red, arrowhead). (H) Small, round mononuclear cells were strongly double-positive for TARC and CD1a (dark brown, arrow). TARC-positive large mononuclear cells were negative for CD68 (red, arrowhead). A through H, original magnification: ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

harvested from both CS-AEP patients and healthy volunteers. These results are in agreement with previous data showing that MDC is produced constitutively by macrophages [30,31]. In contrast, TARC is generated upon activation [21,22,32–34]. TARC expression on BALF cells was observed only in patients with CS-AEP, but not in s-HV. Double staining determined that TARC was strongly expressed in dendritic cells. Although dendritic cells are a

minor component of BALF cells even in CS-AEP, the high expression of TARC by these cells in the inflamed lung might be crucial for the promotion of eosinophilic inflammation in the pathogenesis of CS-AEP. Of course, the contribution of lung structural cells, such as endothelial cells and alveolar epithelial cells, might also be pertinent; however, it is difficult to obtain adequate specimens of these cells from patients with CS-AEP.

There is a limited number studies of the production of CCR4 ligands by primary alveolar macrophages and dendritic cells in lung diseases. Alveolar macrophages from patients with allergic asthma produce MDC and low levels of TARC without any stimulation in vitro [35], but their cytokine regulation is not known. MDC production in monocytes is induced by IL-4 and IL-13 [36,37]. Also, TARC is produced abundantly by monocytes treated with GM-CSF, especially in the presence of IL-4, as well as by dendritic cells derived from monocytes cultured with GM-CSF and IL-4 [32]. In the present study, CS-AEP patients had significantly higher levels of IL-4 and GM-CSF in BALF than did healthy volunteers. It is possible that elevated levels of IL-4 further stimulate MDC and TARC production and promote a positive regulatory loop of additional Th2 cell recruitment. In support of this notion, CCR4 ligand production by adherence-purified BALF cells, which contain alveolar macrophages and dendritic cells [38,39], is upregulated by IL-4. Moreover, increased production of IL-4 and GM-CSF might be important to promote dendritic cell maturation in the initiation of CS-AEP pathogenesis. In contrast, IFN-γ or TNF-α did not augment CCR4 ligand production. IFN-γ suppresses the constitutive expression of MDC in mature macrophages and dendritic cells [37]. In the present study, IFN-γ tended to downregulate CCR4 ligand production by adherence-purified BALF cells; however, this difference was not statistically significant. Higher cell concentrations, higher concentrations of IFN-γ, and longer culture times might be required.

There have been several case reports suggesting that AEP is associated with cigarette smoke; in such cases, a cigarette smoking challenge test typically induces the reappearance of the symptoms [7–11]. Our CS-AEP patients, except for one passive smoker, had started smoking cigarettes within the preceding month and spontaneously improved after cessation of smoking. Consistent with the earlier work, our challenge test induced the symptoms to reappear and to increase the eosinophil proportion in the BALF. Previously, increased IL-4 and IL-5 levels were demonstrated in BALF after a challenge test [9,15], suggesting that cigarette smoke induces a Th2-shifted cytokine profile in CS-AEP pathogenesis. More interestingly, in the present study, challenge with cigarette smoke induced a significant increase in MDC and TARC levels in BALF. Elevation of TARC levels can be explained by high TARC secretion levels by the BALF adherent cells. In contrast, MDC levels in the culture supernatant were almost the same before and 16 h after the challenge, but were elevated compared with those from s-HV. This suggests that MDC and TARC are produced at different time points during the course of CS-AEP. Although only one patient underwent the challenge test, thus limiting statistical analysis, the results obtained indicated that cigarette smoke triggered the production of MDC and TARC. Increased generation of MDC and TARC might contribute to an additional burden of Th2 cells, which induce eosinophil influx. Alternatively, as BALF eosinophils increased quite rapidly after the challenge test, other mechanisms might also be involved. The concentration of eotaxin, a selective chemoattractant for eosinophils, is increased in BALF from patients with eosinophilic pneumonia [40–42]. A recent paper reported that mouse eosinophils migrate in response to TARC [43]. Also, MDC induces dose-dependent chemotaxis of purified human eosinophils [44]. In addition to eotaxin, it is possible that MDC and TARC have direct chemoattractant properties for recruiting eosinophils into the inflamed lung independently from Th2 cytokines.

Recently, Shorr et al. reported epidemiologic investigation of cases of AEP among US military personnel deployed in or near Iraq [45]. Eighteen patients, who all used tobacco, with 78% recently beginning to smoke, were identified among 183,000 military personnel, suggesting rarity of AEP. Regarding association between cigarette smoke and AEP, chemicals including nicotine may alter the Th1/Th2 balance toward Th2 shift, which can lead to expression of chemokines that attract eosinophils and thus induce AEP. Recurrence by cigarette smoke challenge in our patients as well as those presented in previous papers implicates cigarette smoke as a direct cause of AEP. However, given that minority of smokers develop AEP, someone may think that tobacco alone is unlikely to be either a necessary or sufficient condition for the development of CS-AEP. Recent exposure to tobacco may prime the lung in some way such that a second exposure may trigger a cascade of events that culminates in AEP.

In conclusion, CS-AEP patients had extremely high concentrations of MDC and TARC in their BALF. These CCR4 ligands were generated by alveolar macrophages and dendritic cells, which required direct and early contact with extrinsic stimuli, such as cigarette smoke. Indeed, concentrations of MDC and TARC increased after a cigarette smoking challenge test and, more importantly, TARC production by these BALF cells clearly increased in response to exposure to cigarette smoke, implicating cigarette smoke as one of the etiologic factors of CS-AEP. Further studies to elucidate the pathway by which cigarette smoke induces MDC and TARC production are warranted.

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References

[1] J.N. Allen, E. Pacht, J. Gadek, W. Davis, Acute eosinophilic pneumonia as a reversible cause of noninfectious respiratory failure, N. Engl. J. Med. 321 (1989) 569–574.

[2] S. Mehandru, R.L. Smith, G.S. Sidhu, N. Cassai, C.P. Aranda, Migratory pulmonary infiltrates in a patient with rheumatoid arthritis, Thorax 57 (2002) 465–467.

[3] M. Oddo, L. Liaudet, M. Lepori, A.F. Broccard, M.D. Schaller, Relapsing acute respiratory failure induced by minocycline, Chest 123 (2003) 2146–2148.

[4] E. Miyazaki, K. Sugisaki, T. Shigenaga, T. Matsumoto, S. Kita, Y. Inobe, T. Tsuda, A case of acute eosinophilic pneumonia caused by inhalation of Trichosporon terrestre, Am. J. Respir. Crit. Care Med. 151 (1995) 541–543.

[5] S. Imokawa, A. Sato, H. Hayakawa, M. Toyoshima, M. Taniguchi, K. Chida, Possible involvement of an environmental agent in the development of acute eosinophilic pneumonia, Ann. Allergy Asthma. Immunol. 76 (1996) 419–422.

[6] W.N. Rom, M. Weiden, R. Garcia, T.A. Yie, P. Vathesatogkit, D.B. Tse, G. McGuinness, V. Roggli, D. Prezant, Acute eosinophilic pneumonia in a New York City firefighter exposed to World Trade Center dust, Am. J. Respir. Crit. Care Med. 166 (2002) 797–800.

[7] M. Nakajima, T. Manabe, Y. Niki, T. Matsushima, Cigarette smoke-induced acute eosinophilic pneumonia, Radiology 203 (1998) 829–831.

[8] H. Shintani, M. Fujimura, M. Yasui, K. Ueda, S. Kameda, M. Noto, T. Matsuda, M. Kobayashi, Acute eosinophilic pneumonia caused by cigarette smoking, Intern. Med. 39 (2000) 66–68.

[9] Y. Tanino, E. Yamaguchi, K. Takaoka, Y. Fukui, S. Konno, N. Hizawa, M. Nishimura, Cytokines and Th2 cells in AEP of smoking, Allergy 57 (2002) 463–464.

[10] Y. Shiota, T. Kawai, H. Matsumoto, J. Hiyama, Y. Tokuda, M. Marukawa, T. Ono, H. Mashiba, Acute eosinophilic pneumonia following cigarette smoking, Intern. Med. 39 (2000) 830–833.

[11] K. Watanabe, M. Fujimura, K. Kasahara, M. Yasui, S. Myou, T. Kita, A. Watanabe, S. Nakao, Acute eosinophilic pneumonia following cigarette smoking: a case report including cigarette-smoking challenge test, Intern. Med. 41 (2002) 1016–1020.

[12] J.N. Allen, Z. Liao, M.D. Wewers, E.A. Altenberger, S.A. Moore, E.D. Allen, Detection of IL-5 and IL-1 receptor antagonist in bronchoalveolar lavage fluid in acute eosinophilic pneumonia, J. Allergy Clin. Immunol. 97 (1996) 1366–1374.

[13] E. Miyazaki, S. Nureki, T. Fukami, T. Shigenaga, M. Ando, K. Ito, H. Ando, K. Sugisaki, T. Kumamoto, T. Tsuda, Elevated levels of thymus- and activation-regulated chemokine in bronchoalveolar lavage fluid from patients with eosinophilic pneumonia, Am. J. Respir. Crit. Care Med. 165 (2002) 1125–1131.

[14] C. Walker, W. Bauer, R.K. Braun, G. Menz, P. Braun, F. Schwarz, T.T. Hansel, B. Villiger, Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia, Am. J. Respir. Crit. Care Med. 150 (1994) 1038–1048.

[15] S. Ito, H. Taniguchi, Y. Kondo, Y. Mizuno, O. Nishiyama, H. Wakayama, T. Nakagawa, H. Hayashi, K. Yamaki, Positive response to smoking challenge test in a case of acute eosinophilic pneumonia, Nihon Kokyuki Gakkai Zasshi 37 (1999) 424–428.

[16] R. Bonecchi, G. Bianchi, P.P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, F. Sinigaglia, Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s, J. Exp. Med. 187 (1998) 129–134.

[17] T. Imai, D. Chantry, C.J. Raport, C.L. Wood, M. Nishimura, R. Godiska, O. Yoshie, P.W. Gray, Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4, J. Biol. Chem. 273 (1998) 1764–1768.

[18] T. Kakinuma, K. Nakamura, M. Wakugawa, H. Mitsui, Y. Tada, H. Saeki, H. Torii, M. Komine, A. Asahina, K. Tamaki, Serum macrophage-derived chemokine (MDC) levels are closely related with the disease activity of atopic dermatitis, Clin. Exp. Immunol. 127 (2002) 270–273.

[19] J.A. Gonzalo, Y. Pan, C.M. Lloyd, G.Q. Jia, G. Yu, B. Dussault, C.A. Powers, A.E. Proudfoot, A.J. Coyle, D. Gearing, J.C. Gutierrez-Ramos, Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation, J. Immunol. 163 (1999) 403–411.

[20] T. Sekiya, H. Yamada, M. Yamaguchi, K. Yamamoto, A. Ishii, O. Yoshie, Y. Sano, A. Morita, K. Matsushima, K. Hirai, Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics, Allergy 57 (2002) 173–177.

[21] T. Sekiya, M. Miyamasu, M. Imanishi, H. Yamada, T. Nakajima, M. Yamaguchi, T. Fujisawa, R. Pawankar, Y. Sano, K. Ohta, A. Ishii, Y. Morita, K. Yamamoto, K. Matsushima, O. Yoshie, K. Hirai, Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells, J. Immunol. 165 (2000) 2205–2213.

[22] J.J. Campbell, G. Haraldsen, J. Pan, J. Rottman, S. Qin, P. Ponath, D.P. Andrew, R. Warnke, N. Ruffing, N. Kassam, L. Wu, E.C. Butcher, The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells, Nature 400 (1999) 776–780.

[23] C. Vestergaard, H. Yoneyama, M. Murai, K. Nakamura, K. Tamaki, Y. Terashima, T. Imai, O. Yoshie, T. Irimura, H. Mizutani, K. Matsushima, Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions, J. Clin. Invest. 104 (1999) 1097–1105.

[24] K.A. Pulford, E.M. Rigney, K.J. Micklem, M. Jones, W.P. Stross, K.C. Gatter, D.Y. Mason, KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections, J. Clin. Pathol. 42 (1989) 414–421.

[25] K. Micklem, E. Rigney, J. Cordell, D. Simmons, P. Stross, H. Turley, B. Seed, D. Mason, A human macrophage-associated antigen (CD68) detected by six different monoclonal antibodies, Br. J. Haematol. 73 (1989) 6–11.

[26] F. Sotzik, A. Boyd, K. Shortman, Surface antigens of human thymocyte populations defined by CD3, CD4 and CD8 expression: CD1a is expressed by mature thymocytes but not peripheral T cells, Immunol. Lett. 36 (1993) 101–106.

[27] A. Aruffo, B. Seed, Expression of cDNA clones encoding the thymocyte antigens CD1a, b, c demonstrates a hierarchy of exclusion in fibroblasts, J. Immunol. 143 (1989) 1723–1730.

[28] J. Longley, J. Kraus, M. Alonso, R. Edelson, Molecular cloning of CD1a (T6), a human epidermal dendritic cell marker related to class I MHC molecules, J. Invest. Dermatol. 92 (1989) 628–631.

[29] S. Katoh, K. Fukushima, N. Matsumoto, K. Matsumoto, K. Abe, N. Onai, K. Matsushima, S. Matsukura, Accumulation of CCR4-expressing CD4+ T cells and high concentration of its ligands (TARC and MDC) in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia, Allergy 58 (2003) 518–523.

[30] R. Godiska, D. Chantry, C.J. Raport, S. Sozzani, P. Allavena, D. Leviten, A. Mantovani, P.W. Gray, Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells, J. Exp. Med. 185 (1997) 1595–1604.

[31] D. Chantry, A.J. DeMaggio, H. Brammer, C.J. Raport, C.L. Wood, V.L. Schweickart, A. Epp, A. Smith, J.T. Stine, K. Walton, L. Tjoelker, R. Godiska, P.W. Gray, Profile of human macrophage transcripts: insights into macrophage biology and identification of novel chemokines, J. Leukoc. Biol. 64 (1998) 49–54.

[32] T. Imai, M. Nagira, S. Takagi, M. Kakizaki, M. Nishimura, J. Wang, P.W. Gray, K. Matsushima, O. Yoshie, Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine, Int. Immunol. 11 (1999) 81–88.

[33] T. Imai, T. Yoshida, M. Baba, M. Nishimura, M. Kakizaki, O. Yoshie, Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein–Barr virus vector, J. Biol. Chem. 271 (1996) 21514–21521.

[34] F. Sallusto, B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, A. Lanzavecchia, Distinct patterns and kinetics of chemokine production regulate dendritic cell function, Eur. J. Immunol. 29 (1999) 1617–1625.

[35] O. Fahy, S. Senechal, J. Pene, A. Scherpereel, P. Lassalle, A.B. Tonnel, H. Yssel, B. Wallaert, A. Tsicopoulos, Diesel exposure favors Th2 cell recruitment by mononuclear cells and alveolar macrophages from allergic patients by differentially regulating macrophage-derived chemokine and IFN-gamma-induced protein-10 production, J. Immunol. 168 (2002) 5912–5919.

[36] D.P. Andrew, M.S. Chang, J. McNinch, S.T. Wathen, M. Rihanek, J. Tseng, J.P. Spellberg, C.G. Elias III, STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13, J. Immunol. 161 (1998) 5027–5038.

[37] R. Bonecchi, S. Sozzani, J.T. Stine, W. Luini, G. D'Amico, P. Allavena, D. Chantry, A. Mantovani, Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses, Blood 92 (1998) 2668–2671.

[38] J.E. Losa Garcia, F.M. Rodriguez, M.R. Martin de Cabo, M.J. Garcia Salgado, J.P. Losada, L.G. Villaron, A.J. Lopez, J.L. Arellano, Evaluation of inflammatory cytokine secretion by human alveolar macrophages, Mediators Inflamm. 8 (1999) 43–51.

[39] C. Jancic, H.E. Chuluyan, A. Morelli, A. Larregina, E. Kolkowski, M. Saracco, M. Barboza, W.S. Leiva, L. Fainboim, Interactions of dendritic cells with fibronectin and endothelial cells, Immunology 95 (1998) 283–290.

[40] P.D. Ponath, S. Qin, D.J. Ringer, I. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J.-A. Gonzalo, W. Newman, J.-C. Guiterrez-Ramos, C.R. Mackay, Cloning of human eosinophil chemoattractant eotaxin, J. Clin. Invest. 73 (1996) 604–612.

[41] P.D. Panath, S. Qin, T.W. Post, J. Wang, L. Wu, N.P. Gerad, W. Newman, C. Gerard, C.R. Mackay, Molecular cloning and characterization of a human eotaxin receptor expressed on eosinophils, J. Exp. Med. 183 (1996) 2437–2448.

[42] S. Katoh, N. Matsumoto, K. Fukushima, H. Mukae, J.I. Kadita, S. Kohno, S. Matsukura, Elevated chemokine levels in bronchoalveolar lavage fluids of patients with eosinophilic pneumonia, J. Allergy Clin. Immunol. 106 (2000) 730–736.

[43] M.T. Borchers, T. Ansay, R. DeSalle, B.L. Daugherty, H. Shen, M. Metzger, N.A. Lee, J.J. Lee, In vitro assessment of chemokine receptor–ligand interactions mediating mouse eosinophil migration, J. Leuko. Biol. 71 (2002) 1033–1041.

[44] B.S. Bochner, C.A. Bickel, M.L. Taylor, D.W. MacGlashan Jr., P.W. Gray, C.J. Raport, R. Godiska, Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3- and CC chemokine receptor 4-independent manner, J. Allergy Clin. Immunol. 103 (1999) 527–532.

[45] A.F. Shorr, S.L. Scoville, S.B. Cersovsky, G.D. Shanks, C.F. Ockenhouse, B.L. Smoak, W.W. Carr, B.P. Petruccelli, Acute eosinophilic pneumonia among US military personnel developed in or near Iraq, JAMA 292 (2004) 2997–3005.

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Reflex Seizures in Patients with Malformations of Cortical Development and Refractory Epilepsy

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Summary: *Purpose:* Malformations of cortical development (MCDs) are usually highly epileptogenic, and their hyperexcitability could facilitate the occurrence of reflex seizures. We sought to characterize reflex seizures in patients with MCDs and refractory epilepsy.

Methods: Clinical, electrographic, and neuroimaging data were reviewed in eight patients with MCDs who had reflex seizures reproduced during presurgical evaluation.

Results: All eight patients had both reflex and spontaneous seizures. In six, however, drop attacks or axial myoclonic seizures occurred only upon specific sensory stimulation. Reflex seizures were induced by more than one type of stimulus in most patients, but anatomofunctional correlations could usually be invoked. Six patients had significant intellectual impairment. Surgical resection controlled seizures in two patients.

Conclusions: Reflex seizures in patients with MCDs may be medically refractory and may often manifest as drop attacks or axial myoclonus. Surgical resection of focal lesions can bring reflex seizures under control. Putative mechanisms related to the relatively low frequency of reflex seizures in MCDs are discussed. **Key Words:** Reflex seizures—Malformations of cortical development—High degrees of epileptogenicity.

Reflex seizures most commonly occur in patients with genetically related epilepsy syndromes (1–6), in which triggering stimuli are related to photosensitivity or higher-order cognitive processing (7,8). Seizure provocation through other stimuli and in patients with symptomatic epilepsies is less common, although perirolandic lesions are known to induce reflex seizures.

A common assumption regarding reflex seizures is that hyperexcitable intracortical or corticosubcortical sensory circuits lead to fast and uninhibited recruiting and synchronization of neuronal pools (9–11). Converging clinical and experimental evidence suggests that malformations of cortical development (MCDs) are among the most hyperexcitable epileptogenic lesions (12,13). In addition, these malformations are often intrinsically epileptogenic (14–16) and may establish abnormal connections with other cortical and subcortical structures (17,18). Thus, patients with MCDs are prone to respond with abnormally increased electrical volleys to modality-specific afferent stimuli, which would facilitate reflex provocation of seizures. Nonetheless, *series* of patients with MCDs and reflex seizures are not found in the literature. We report eight patients with reflex seizures associated with MCDs confirmed by imaging, histopathology, or both.

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METHODS

Eight patients (four men) with spontaneous and reproducible reflex seizures and a MCD were studied. We defined specific reflex seizure precipitants as any sensory stimulus that precipitated the seizures in daily life and could be confirmed during evaluation. Self-precipitation was defined as recurrent intentional self-provocation of seizures through a specific type of sensory stimulation. Patients reported herein were recruited from a pool of 30 to 100 patients with MCDs evaluated in each of the