Table 1. MICs of anti-pneumococcal agents

Anti-pneumococcal agent	MIC (μg/ml) of PSSP (n = 16)			MIC (μg/ml) of PISP + PRSP (n = 26)		
	Range	50%	90%	Range	50%	90%
Benzylpenicillin	≦0.06	≦0.06	≦0.06	0.13–2	1	2
Ampicillin	≦0.06–0.13	≦0.06	0.13	0.13–4	1	2
Cefotiam	≦0.06–4	0.25	0.5	0.5–16	4	8
Cefepime	≦0.06–1	0.25	0.5	0.5–2	1	1
Cefditoren	≦0.06–0.5	≦0.06	0.13	≦0.06–1	0.25	0.25
Faropenem	≦0.06	≦0.06	≦0.06	≦0.06–0.5	0.25	0.25
Panipenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13	≦0.06	0.13
Biapenem	≦0.06	≦0.06	≦0.06	≦0.06–0.25	0.13	0.13
Erythromycin	≦0.125–>16	4	>16	≦0.125–>16	4	>16
Clarithromycin	≦0.125–>16	4	>16	≦0.125–>16	2	>16
Azithromycin	0.25–>128	128	>128	0.5–>128	16	>128
Telithromycin	0.013–0.06	0.06	0.06	0.013–0.25	0.06	0.06
Ciprofloxacin	0.25–2	0.5	1	0.25–1	0.5	0.5
Levofloxacin	0.5–1	0.5	1	0.25–1	0.5	1
Gatifloxacin	0.13–0.5	0.25	0.25	0.13–0.25	0.25	0.25

Table 2. Correlation between penicillin resistance and genetic characteristics

Genetic characteristic	PSSP (n = 16)		PISP + PRSP (n = 26)	
	g-PSSP (n = 6)	g-PISP (n = 10)	g-PISP (n = 3)	g-PRSP (n = 23)
No alteration	6			
pbp2x		8		
pbp1a + *pbp2x*		2	2	
pbp2x + *pbp2b*			1	
pbp1a + *pbp2x* + *pbp2b*				23
None	3	2	1	
mefA	2	2		12
ermB	1	5	1	11
mefA + *ermB*		1	1	

Collection). The results were interpreted according to the present NCCLS standards.[4] We reviewed these samples for alterations of penicillin-binding proteins and genetic transmission of macrolide tolerance. The genes reviewed were *pbp1a*, *pbp2x*, *pbp2b*, *mefA*, and *ermB*. Variations in the genes for penicillin-binding protein and macrolide resistance determinants were screened by PCR, using methods reported previously.[5] In the NCCLS recommendations, an MIC of penicillin of ≦0.06 μg/ml is classified as PSSP, an 0.12 ≦ MIC ≦ 1.0 μg/ml is classified as PISP, and an MIC of 2.0 μg/ml ≦ is classified as PRSP. Regarding resistant gene abnormalities, we defined no alteration as genetic-PSSP (g-PSSP); abnormalities of *pbp2x* only, or *pbp1a* + *pbp2x*, or *pbp2x* + *pbp2b* as g-PISP; and abnormalities of *pbp1a* + *pbp2x* + *pbp2b* as g-PRSP.

The predominant source of isolation was sputum from pediatric patients with pneumonia (n = 27), followed by sputum or nasopharynx specimens from adults with acute respiratory infection (n = 9), otorrhea from otolaryngology patients with acute otitis media (n = 3), cerebrospinal fluid from a pediatric patient with purulent meningitis (n = 1), and blood with sepsis from an internal medicine patient (n = 1).

Table 1 lists comparative antimicrobial activities against *S. pneumoniae*. In PISP + PRSP strains, the MIC$_{90}$ of seven β-lactams (ampicillin, cefotiam, cefepime, cefditoren, faropenem, panipenem, and biapenem) were twofold or higher than those in PSSP strains. For macrolides and fluoroquinolones, however, the MIC$_{90}$ values were almost equal between the two groups. Results of PCR investigation for gene alterations and the MIC distributions of benzylpenicillin and macrolide resistance determinants are shown in Table 2. Twenty-three strains exhibited genetic variations at *pbp1a* + *pbp2x* + *pbp2b*, which were g-PRSP. Detection of the *mefA* gene or the *ermB* gene is shown in the lower part of Table 2. Two strains exhibited genetic variations at *mefA* + *ermB*. There was no correlation between *pbp* mutations and the existence of the *mefA* gene or the *ermB* gene. Genetic resistance and the MIC ranges of β-lactams are shown in Table 3. The g-PRSP strains showed higher MIC values compared with the g-PSSP or g-PISP strains. The existence of a triple abnormal *pbp* gene affected drug resistance, not only in penicillin but also in cephems or carbapenems. Correlation between *mefA* or *ermB* and macrolide resistance was: only *mefA* was found in 16 isolates, for which the MIC range was 0.5–>16 μg/ml for erythromycin and clarithromycin, and 2–>128 μg/ml for azithromycin. Only *ermB* was found in 18 isolates, for which the MIC range was 2–>16 μg/ml for erythromycin and clarithromycin, and 16–>128 μg/ml for azithromycin. Two

Table 3. Correlation between *pbp* alterations and β-lactam resistance

Anti-pneumococcal agent	MIC range (μg/ml)			
	PSSP (n = 16)		PISP + PRSP (n = 26)	
	g-PSSP (n = 6)	g-PISP (n = 10)	g-PISP (n = 3)	g-PRSP (n = 23)
Benzylpenicillin	≦0.06	≦0.06	0.25–0.5	0.13–2
Ampicillin	≦0.06	≦0.06–0.13	0.13–0.5	0.5–4
Cefotiam	0.13–0.25	≦0.06–4	0.5–1	1–16
Cefepime	≦0.06–0.13	0.25–1	0.5–1	0.5–2
Cefditoren	≦0.06	≦0.06–0.5	0.13–0.5	≦0.06–1
Faropenem	≦0.06	≦0.06	≦0.06	≦0.06–0.5
Panipenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13
Biapenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13

isolates had both *mefA* and *ermB*, and the MIC ranges for these two isolates were ≧16 μg/ml for erythromycin and clarithromycin, and >128 μg/ml for azithromycin.

A limitation of this study is that there was a small number of pneumococcal strains within a short term, and the study took place in a single medical hospital. Under these conditions, we demonstrated that g-PISP and g-PRSP accounted for 11.5% (3/26) and 88.5% (23/26) of PISP + PRSP, respectively. Ubukata et al.[2] observed that genotypically proven g-PISP and g-PRSP had been isolated in 2002 at the rates of 33.0% and 54.9%, respectively. Pediatric strains have been shown, worldwide, to be more resistant than those from adults,[6] and household transmission of *S. pneumoniae* has been observed;[7] therefore, we should be cautious about the increase of drug-resistant *S. pneumoniae* in adults. The oral β-lactams cefditoren and faropenem showed favorable antibacterial activities, with MIC_{90} values of 0.25 μg/ml against PISP + PRSP; therefore, they will be useful for empiric therapy against pneumococcal infections. The macrolide resistance mechanisms, *mefA* and *ermB*, were accurately detected and correlated with the MICs of erythromycin, clarithromycin, and azithromycin. The existence of both *mefA* and *ermB* confers strong resistance in macrolides. Telithromycin, however, was very effective against PISP + PRSP; therefore it will be useful for severe pediatric pneumococcal infection. Newer fluoroquinolones, such as gatifloxacin, have much greater in vitro activities with lower MICs against *S. pneumoniae*;[8] however, increasing resistance to fluoroquinolones has been documented in Hong Kong and Canada.[9,10] In Japan, the prevalence of fluoroquinolone-resistant *S. pneumoniae* is thought to be very low.[11] Yokota et al.[12] reported seven quinolone-resistant strains among 293 clinical isolates during 3 years. Although we did not find a quinolone-resistant strain in this study, we should follow strains for quinolone susceptibility for a longer period of time.

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ELSEVIER

Cytokines Involved in CNS Manifestations Caused by *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae **sometimes causes central nervous system manifestations, which may involve the host immune response, as the organism does not directly damage neural cells, or release toxins. Therefore we measured the levels of interleukin-6, interleukin-8, interleukin-18, interferon-γ, tumor necrosis factor-α, and transforming growth factor-β_1 in serum and cerebrospinal fluid samples from patients who manifested central nervous system manifestations during acute** ***M. pneumoniae*** **infection. The subjects were nine patients with early-onset encephalitis (central nervous system disease onset within 7 days from the onset of fever), four with late-onset encephalitis (onset at 8 days or later), three with encephalitis but without fever, and three with aseptic meningitis. Intrathecal elevations of interleukin-6 and interleukin-8 in all four types of central nervous system manifestations, and of interleukin-18 in late-onset encephalitis were observed. None of the cerebrospinal fluid samples contained detectable levels of interferon-γ, tumor necrosis factor-α, or transforming growth factor-β_1. In conclusion, interleukin-6, interleukin-8, and interleukin-18 might be involved in the inflammatory process leading to the central nervous system manifestations caused by** ***M. pneumoniae.*** **© 2005 by Elsevier Inc. All rights reserved.**

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Introduction

It is well recognized that *Mycoplasma pneumoniae* can cause central nervous system manifestations, but the underlying pathomechanisms remain unclear. In this context, many studies have demonstrated that *M. pneumoniae* can be isolated, or its genome can be detected by polymerase chain reaction, in cerebrospinal fluid samples from patients with central nervous system manifestations. Therefore, direct invasion of the central nervous system by this organism seems to be a prerequisite for the development of central nervous system manifestations. On the other hand, there is no evidence that *M. pneumoniae* can directly damage neural cells, so immune-mediated pathomechanisms may be involved. If so, inflammatory cytokines are likely candidates for inflammatory mediators.

In previous studies, we found that the *M. pneumoniae* genome was detectable by polymerase chain reaction at a significantly higher rate in cerebrospinal fluid samples from patients with early-onset encephalitis (defined as central nervous system disease onset within 7 days from the onset of fever) than in cerebrospinal fluid from patients with late-onset encephalitis (onset at 8 days or later) [1,2]. Subsequent research confirmed this finding [3,4]. Thus, it seems that there may be distinct types of neurologic complications due to *M. pneumoniae* infection. The present study was conducted to determine whether various cytokines are specifically associated with the central nervous system manifestations caused by *M. pneumoniae*.

Patients and Methods

Patients

Characteristics of the patients, who were examined during the 5 years of 1999-2003, are summarized in Table 1. Diagnosis of *M. pneumoniae* acute infection was made serologically based on the following criteria: (1) a fourfold or greater rise in antimycoplasmal antibody titer measured by complement fixation or microparticle-agglutination test, or (2) a highest titer of 1:256 or more by complement fixation or 1:320 or more by microparticle-agglutination test. Cases were excluded when coinfection with another agent was apparent. The final decisions made by the attending physicians were respected concerning the diagnosis of encephalitis. Treatment was various, and antibiotics directed against *M. pneumoniae* were not used as an initial treatment in most of the cases. None of the patients died. Fifteen patients achieved full recovery within 28

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Table 1. Characteristics of patients

Case No.	Age (yr)	Sex	Mp-antibody*	Pneumonia†	Type
1	10	F	1:5120	−	E, enc
2	15	F	1:1280	+	E, ADEM
3	9	F	1:1280	+	E, mg/enc
4	8	M	1:256	−	E, mg/enc
5	6	M	1:2560	+	E, enc/cerebellitis
6	7	M	>1:1280	−	E, mg/enc
7	12	M	1:10,240	−	E, ADEM
8	4	F	1:8 to 1:64	−	E, enc
9	2	M	1:2560	+	E, enc
10	7	M	1:4096	+	L, mg/enc
11	6	M	>1:20,480	+	L, mg/enc
12	2	M	1:1280	+	L, enc
13	6	F	1:320	−	L, ADEM
14	9	M	1:10,240	−	No, mg/enc
15	14	F	1:80 to 1:640	−	No, enc/GBS
16	10	F	1:2560	−	No, mg/enc
17	10	M	1:2048	−	mg
18	8	M	1:1024	−	mg
19	9	F	1:5120	+	mg

* *M. pneumoniae* antibody was measured by using a microparticle agglutination test, except in cases 4, 8, 10, 17, and 18, where a complement fixation test was used.
† +, present; −, absent.

Abbreviations:
ADEM = Acute disseminated encephalomyelitis
E = Early-onset encephalitis
enc = Encephalitis
GBS = Guillain-Barré syndrome
L = Late-onset encephalitis
mg = Meningitis
mg/enc = Meningoencephalitis
No = Encephalitis without fever

days. Four patients had minimal remaining neurologic deficits, which affected their daily activities to some degree, such as mild hearing loss, mild spasticity, or exacerbation of seizure activity in a patient with epileptic illness.

Cytokine Assays

Previous studies have revealed that various kinds of inflammatory and immunomodulatory cytokines are involved in the development of central nervous system manifestations caused by bacterial and other infections. Among them, we focused on interferon (IFN)-γ [5], tumor necrosis factor (TNF)-α [6], interleukin (IL)-6 [7,8], and IL-8 [9], because these cytokines have been reported to be involved in the pathogenesis of central nervous system manifestations by other organisms and can also be induced by *M. pneumoniae* [10,11]. In addition, we focused on IL-18 because we and our associates had recently found that local production of IL-18 and IL-8 in the lung plays a significant role in the pathogenesis of pulmonary disease caused by *M. pneumoniae* [12,13]. Moreover, transforming growth factor (TGF)-β_1 was included because recent studies have demonstrated that TGF-β_1 might work as a regulatory factor for central nervous system diseases through its antagonistic function, e.g., for IL-8 [14]. Levels of these cytokines were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits; the properties of the ELISA systems are summarized in Table 2.

Results

All results are presented in Table 3. Sequential serum or cerebrospinal fluid samples were obtained at intervals of 4 to 14 days from some patients. Because IL-18 and TGF-β_1 have wide physiologic concentration ranges between the detection limit and the normal upper limit (12.5 pg/mL to 260 pg/mL for IL-18, and 4.7 ng/mL to 74 ng/mL for

Table 2. Properties of ELISA kits for cytokine determination

Cytokine	Manufacturer	Detection Limit	Normal Range
IL-6	Fuji Rebio (Japan)	2.5	<10.0
IL-8	Amersham (UK)	5.0	<10.0
IL-18	MBL (Japan)	12.5	<260
IFN-γ	Amersham	0.1	<1.5
TNF-α	Amersham	4.5	<7.5
TGF-β_1	Amersham	4.7	<74

Detection limits are according to the manufacturer's instructions, and normal ranges are based on the manufacturer's instructions and our own previous results. These values relate to serum, and are here tentatively assumed to be applicable to cerebrospinal fluid. Values are expressed in pg/mL except TGF-β_1, in ng/mL.

Abbreviation:
ELISA = Enzyme-linked immunosorbent assay
IFN-γ = Interferon gamma
IL = Interleukin
TGF-β_1 = Transforming growth factor beta-1
TNF-α = Tumor necrosis factor alpha

Table 3a. Results of cytokine determination in serum and CSF samples from patients with CNS manifestations caused by *M. pneumoniae*

Case	Sample*	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β_1
1 (E, enc)	Serum-1	NT	NT	<260	1.5	<4.5	<74
	CSF-1	NT	NT	<260	<1.5	<4.5	<4.7
2 (E, ADEM)	Serum-1	12.8	<5.0	319	<1.5	<4.5	103.4
	CSF-1	91.7	155.8	<260	<1.5	<4.5	<4.7
3 (E, mg/enc)	Serum-1	8.9	<5.0	528	10.6	<4.5	<74
	Serum-2	597	1547	271	NT	<4.5	<74
	CSF-1	31.9	363.4	<260	<1.5	<4.5	<4.7
	CSF-2	7.3	<5.0	<260	<1.5	<4.5	<4.7
4 (E, mg/enc)	Serum-1	12.8	<5.0	<260	<1.5	<4.5	90.2
	CSF-1	7.7	6.5	<260	<1.5	<4.5	<4.7
5 (E, enc/cerebellitis)	Serum-1	8.0	NT	281	<1.5	<4.5	89.0
	Serum-2	7.0	NT	288	NT	<4.5	<74
	CSF-1	13.4	NT	<260	<1.5	<4.5	<4.7
6 (E, mg/enc)	Serum-1	9.6	<5.0	315	<1.5	<4.5	<74
	Serum-2	NT	NT	316	<1.5	<4.5	<74
	CSF-1	17.9	62.1	<260	NT	<4.5	<4.7
7 (E, ADEM)	Serum-1	11.2	<5.0	268	<1.5	<4.5	<74
	CSF-1	8.6	169.4	NT	<1.5	<4.5	<4.7
8 (E, enc)	Serum-1	NT	9.0	335	<1.5	<4.5	NT
	CSF-1	<2.5	42.6	<260	<1.5	<4.5	NT
9 (E, enc)	Serum-1	46.3	137.3	1180	16.9	<4.5	<74
	CSF-1	10.9	224.1	319	<1.5	<4.5	<4.7
10 (L, mg/enc)	Serum-1	7.0	21.3	357	<1.5	<4.5	<74
	CSF-1	7.7	<5.0	<260	<1.5	<4.5	<4.7
	CSF-2	7.7	<5.0	306	<1.5	<4.5	<4.7
	CSF-3	8.9	<5.0	<260	<1.5	<4.5	<4.7

Abbreviations as in Table 3b.

TGF-β_1), values within the physiologic range are reported as <260 pg/mL for IL-18 and <74 ng/mL for TGF-β_1 in Table 3.

Table 4 presents results in cytokine determination for cerebrospinal fluid, which can be considered essentially more important than results for serum, in the survey for the pathomechanism of central nervous system manifestations. In summary, elevations of IL-6 and IL-8 were frequently observed in all types of central nervous system manifestations, whereas IL-18 was rather specifically elevated in late-onset encephalitis. Otherwise, IFN-γ, TNF-α, and TGF-β_1 were not elevated in any sample.

Discussion

We and our associates previously demonstrated that IL-6 was only sporadically elevated in serum, whereas cerebrospinal fluid levels were highly elevated among patients with bacterial meningitis (49,017 ± 44,730 pg/mL), moderately elevated among patients with aseptic meningitis (1076 ± 1572 pg/mL), and somewhat elevated among patients with encephalitis (409 ± 835 pg/mL) [8]. In this context, the results of the present study indicate that the central nervous system manifestations caused by *M. pneumoniae* are rather similar to those caused by viruses rather than those caused by bacteria in terms of IL-6 response. Two of the three cases with aseptic meningitis exhibited moderately elevated levels of cerebrospinal fluid IL-6 (734 pg/mL in Case 18 and 1327 pg/mL in Case 19), and minimal elevations of cerebrospinal fluid IL-6 were found for the patients with encephalitis. Intrathecal production of IL-6 must be associated with central nervous system manifestations caused by *M. pneumoniae*, and might be a rather nonspecific phenomenon resulting from inflammation in the central nervous system rather than having a specific relationship to *M. pneumoniae*.

With regard to IL-8, previous studies have revealed that intrathecal production of this cytokine has a significant presence during the development of central nervous system diseases caused by various infections [9,15,16]. In this study, elevated cerebrospinal fluid levels of IL-8 were present in a total of 14 cases, that is, 82% of the cases tested. In accordance with previous studies [15,16], IL-8 was not detected or was detected at lower levels in serum in most of the cases. Furthermore in Case 3, an early rise of cerebrospinal fluid IL-8 in the absence of IL-8 in serum (acute phase) was followed by a later rise of serum IL-8 (convalescent phase, 10 days later) in the absence of IL-8 in cerebrospinal fluid. These observations strongly suggest that intrathecal production of IL-8 appears to be associated with the central nervous system manifestations caused by *M. pneumoniae*, although it may not be specific to *M. pneumoniae* as in the case of IL-6.

Slightly but consistently elevated levels of cerebrospinal fluid IL-18 were observed in all four patients with late-onset encephalitis. Although the increase of serum IL-18 levels during mycoplasmal infection was not specific to patients with central nervous system manifesta-

Table 3b. **Results of cytokine determinations in serum and CSF samples from patients with CNS manifestations caused by *M. pneumoniae***

Case	Sample*	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β_1
11 (L, mg/enc)	Serum-1	23.0	<5.0	1005	<1.5	<4.5	86.9
	CSF-1	64.5	430.6	331	<1.5	<4.5	<4.7
12 (L, enc)	Serum-1	NT	246.0	622	66	<4.5	<74
	Serum-2	NT	104.8	1240	38	<4.5	<74
	Serum-3	NT	10.5	305	<1.5	<4.5	78.2
	CSF-1	NT	114.0	285	<1.5	<4.5	<4.7
13 (L, ADEM)	Serum-1	19.2	7.0	506	<1.5	<4.5	121.7
	CSF-1	1259	147.9	282	<1.5	<4.5	<4.7
14 (No, mg/enc)	Serum-1	7.3	<5.0	<260	<1.5	<4.5	74.3
	CSF-1	<2.5	<5.0	<12.5	<1.5	<4.5	<4.7
15 (No, enc/GBS)	Serum-1	11.2	28.3	<260	<1.5	<4.5	102.3
	CSF-1	9.6	201.4	264	<1.5	<4.5	<4.7
16 (No, mg/enc)	Serum-1	8.9	<5.0	398	<1.5	<4.5	74.4
	CSF-1	142	327.4	336	<1.5	<4.5	<4.7
	CSF-2	226	NT	<260	<1.5	<4.5	<4.7
17 (mg)	Serum-1	8.3	119.3	<260	<1.5	<4.5	73.9
	CSF-1	30.7	219.3	<260	<1.5	<4.5	<4.7
	CSF-2	7.0	<5.0	<12.5	<1.5	<4.5	<4.7
18 (mg)	Serum-1	10.9	244.8	478	<1.5	<4.5	95.8
	CSF-1	734	252.2	<260	<1.5	<4.5	<4.7
19 (mg)	Serum-1	8.0	<5.0	NT	NT	<4.5	<74
	CSF-1	1327	1037	<260	<1.5	<4.5	<4.7

Values are expressed in pg/mL except TGF-β_1, in ng/mL.
* Samples with the same number were obtained on the same occasion in each case.

Abbreviation:

ADEM	= Acute disseminated encephalomyelitis	L	= Late-onset encephalitis
CSF	= Cerebrospinal fluid	mg	= Meningitis
E	= Early-onset encephalitis	mg/enc	= Meningoencephalitis
enc	= Encephalitis	No	= Encephalitis without fever
GBS	= Guillain-Barré syndrome	NT	= Not tested
IFN	= Interferon gamma	TGF-β_1	= Transforming growth factor beta-1
IL	= Interleukin	TNF-α	= Tumor necrosis factor alpha

tions [12], elevation of cerebrospinal fluid IL-18 was rather specifically associated with late-onset encephalitis in this study. Recent studies have revealed that IL-18 plays a significant role in the pathomechanism of murine experimental autoimmune encephalomyelitis [17]. It is possible that the increased level of cerebrospinal fluid IL-18 irrespective of whether it is produced intrathecally or not, plays a role in the pathomechanism of late-onset encephalitis, which is likely to be immune-mediated.

With regard to IFN-γ and TNF-α, the results of this study indicate that these cytokines do not play a role in the pathomechanism of central nervous system manifestations caused by *M. pneumoniae*. It also seems unlikely that TGF-β_1 has any role in the central nervous system manifestations.

We recently reported that the production of IL-18 in the systemic circulation and more importantly in the lung has a significant role in the pathomechanism of pulmonary disease caused by *M. pneumoniae* in children [12], as well as in adults [18]. The role of IL-18 in this case presumably involves induction of T helper type 1 cytokines and possibly IL-8 [13], whereas that of IL-8 may be to recruit neutrophils to the alveolar spaces. Although IL-8 may function to recruit neutrophils to the central nervous system in connection with the central nervous system manifestations, we found no correlation between the levels of cerebrospinal fluid IL-8 and the cerebrospinal fluid neutrophil cell counts (data not shown). That function of IL-8 might be restricted in a particular microenvironment in the central nervous system and thus might not be

Table 4. **Summary of results in cytokine determination for cerebrospinal fluid**

	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β_1
Early-onset encephalitis	5/8	6/7	1/8	0/8	0/9	0/8
Late-onset encephalitis	2/3	3/4	4/4	0/4	0/4	0/4
Encephalitis without fever	1/3	2/3	1/3	0/3	0/3	0/3
Aseptic meningitis	3/3	3/3	0/3	0/3	0/3	0/3

No. of cases beyond the normal range/No. tested.
Abbreviations as in Table 3.

reflected in the cerebrospinal fluid cell counts. On the other hand, although the levels of IL-18 and IL-8 in the lung are closely associated with the pulmonary disease severity [12,13,18], none of the above factors appears to be closely associated with the clinical manifestations of central nervous system diseases, that is, the type of encephalitis, the severity of acute-phase clinical symptoms, or the neurologic outcome. In this respect, these cytokines may not play a central role in the central nervous system and there may be some other factors that are more closely associated with central nervous system manifestations.

Mycoplasmas have biologic activities which involve many aspects of host–pathogen interaction, and there has been a long-standing controversy about the use of corticosteroids to treat central nervous system manifestations caused by *M. pneumoniae* [19]. In the present study, unfortunately, treatment was not controlled, and we believe that not a few cases recovered with a self-limited course. The present study demonstrates that several cytokines are frequently expressed in the central nervous system in patients with *M. pneumoniae* infection. In this respect, recent accumulation of data has confirmed that macrolides have suppressive actions for these inflammatory cytokines in addition to their antimicrobial activity [20]. The anti-inflammatory activities are reported to work under lower concentrations of macrolides when compared with therapeutic ranges for antimicrobial activity. This fact may merit the use of macrolides of low permeability into the central nervous system for the treatment of central nervous system involvement. Taken together, our findings suggest that the routine antimicrobial treatment for *M. pneumoniae* using macrolides must be sufficient for most cases of central nervous system involvement associated with *M. pneumoniae* infection and lend support to the idea that the use of corticosteroids with concomitant administration of antibiotics would be effective to treat selected, severe cases of central nervous system manifestations.

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Prospective Surveillance of Community-Onset and Healthcare-Associated Methicillin-Resistant *Staphylococcus aureus* Isolated from a University-Affiliated Hospital in Japan

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***Abstract*: We conducted a prospective comparative study of community-onset (CO) and healthcare-associated (HA) methicillin-resistant *Staphylococcus aureus* (MRSA) strains between 2000 and 2001 at Tokyo Women's Medical University Hospital (1,500 beds) in Japan. Of the 172 consecutive MRSA isolates analyzed, 13 (8%) were categorized as CO-MRSA. The mean age of patients with CO-MRSA was significantly younger than that of patients with HA-MRSA. Most CO-MRSA strains were isolated from skin and more likely to be susceptible to erythromycin, clindamycin, tetracycline, levofloxacin, and spectinomycin compared to HA-MRSA isolates. Pulsed-field gel electrophoresis (PFGE) analysis, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and multi-locus sequence typing (MLST) revealed that CO-MRSA strains were divided into the following multi-clones: 3 clone A: II: ST5 (PFGE type: SCC*mec* type: MLST sequence type); 1 L: II: ST5; 1 H: IV: ST1; 1 I: IV: ST81; 2 D: IV: ST8; 1 B: IV: ST89; 1 B: IV: ST379; and 3 B: IV: ST91. Of the 159 HA-MRSA strains, 124 (78%) belonged to a single clone (PFGE clone A: SCC*mec* type II: *tst* and *sec* positive: coagulase type II: multi-drug resistance). Four CO-MRSA strains belonging to PFGE clone B: SCC*mec* type IV: MLST clonal complex 509 (ST89, 91, 379) had the exfoliative toxin B (*etb*) genes, but all CO-MRSA and HA-MRSA strains did not possess the Panton-Valentine leukocidin (*pvl*) genes. These results demonstrate that multiple lineages of CO-MRSA have the potential for dissemination in the community in Japan.**

***Key words*: Community-onset methicillin-resistant *Staphylococcus aureus* (CO-MRSA), Healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA), Staphylococcal cassette chromosome *mec* (SCC*mec*), Multi-locus sequence typing (MLST)**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens worldwide (5, 32). In Japan, the incidence (60–70%) of MRSA in inpatients continues to remain high (18, 19). Most healthcare-associated MRSA (HA-MRSA) isolates in Japan produce toxic shock syndrome (TSS) toxin-1 (TSST-1) and staphylococcal enterotoxin C (SEC), classified as the superantigen family, and belong to coagulase type II, *mecA*-Tn*554* polymorph I-A, staphylococcal chromosome *mec* (SCC*mec*) type II, and multi-locus sequence type (MLST) ST5 (2, 18–20). MRSA strains with TSST-1 are associated with TSS as

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Abbreviations: BAL, broncho-alveolar lavage; CC, clonal complex; CLI, clindamycin; CNS, coagulase-negative staphylococci; CO-MRSA, community-onset methicillin-resistant *Staphylococcus aureus* (MRSA); ERY, erythromycin; ETA, exfoliative toxin A; ETB, exfoliative toxin B; GEN, gentamicin; HA-MRSA, healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA); LVX, levofloxacin; MDR, multi-drug resistance; MET, methicillin; MIC, minimal inhibitory concentration; MLST, multi-locus sequence typing; MSSA, methicillin-susceptible *Staphylococcus aureus*; NCCLS, National Committee for Clinical Laboratory Standards; NICU, neonatal intensive care unit; NTED, neonatal TSS-like exanthematous diseases; OXA, oxacillin; PFGE, pulsed-field gel electrophoresis; PVL, Panton-Valentine leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*; SEA to SEE, staphylococcal enterotoxin A to E; SPT, spectinomycin; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; TSA, Trypticase-Soy agar; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin-1; TWMUH, Tokyo Women's Medical University Hospital; VAN, vancomycin.

puerperal infection (25) and neonatal TSS-like exanthematous diseases (NTED) (37, 38). Recently, NTED has been reported in France (41). This major, new clone spread during the 1990s in Japan, and nationwide dissemination of this MRSA clone, especially in neonatal intensive care units (NICU), has been associated with pandemic NTED in Japan (18, 19).

Recently, MRSA has emerged in patients without established risk factors in the community (4–6, 10, 26, 32, 34). Community-onset MRSA (CO-MRSA) is predominantly associated with skin and soft tissue infections (10, 23, 26, 32), although life-threatening pneumonia has been reported (23). CO-MRSA strains are characterized by lack of multi-drug resistance (4–6, 8, 9, 26, 32), predominance of SCC*mec* type IV (4, 6, 8, 9, 26, 28, 30, 42, 43) and frequent possession of the Panton-Valentine leukocidin (*pvl*) genes (22, 23, 26, 42, 43).

The incidence of MRSA in outpatients has increased to 20–40% of *S. aureus* strains (18, 19). The isolation rate of MRSA among *S. aureus* in outpatients in our hospital has increased to 30% of all *S. aureus* isolates (18). There have been few reports on the clinical characteristics of CO-MRSA infections as well as phenotypic and genetic characterization of CO-MRSA strains as compared with HA-MRSA in Japan. From a global control and prevention perspective, it is important to study the molecular epidemiology of CO-MRSA strains in Japan. In the present study, we conducted a prospective surveillance of CO-MRSA and HA-MRSA strains in our university-affiliated hospital. In addition, a phenotypic and genetic characterization of these strains was performed.

Materials and Methods

Study design. We prospectively collected 200 consecutive MRSA strains (100 strains from inpatients and 100 strains from outpatients) isolated from various sources between May 24, 2000 and Feb. 19, 2001 at Tokyo Women's Medical University Hospital (TWMUH, 1,500 beds). Only the first isolate from each patient was analyzed. The medical record of each patient with MRSA was reviewed to determine whether it met the case definition of community-onset or healthcare-associated. The case definition of CO-MRSA was a patient who had no history of hospitalization in the previous 6 months or admission to a long-term care facility, surgery, dialysis, endotracheal intubation, placement of an indwelling intravenous or urinary catheter, injection drug use, diabetes, neoplasm, chronic renal failure, chronic liver diseases, corticosteroid therapy and anticancer chemotherapy (34, 43). A community-onset case was defined if MRSA was isolated from cultures of specimens collected within 72 hr prior to admission to our hospital (34, 43). MRSA was considered to be healthcare-associated in all cases that were not community-onset.

Bacterial strains. S. aureus was identified by standard microbiological methods including Gram staining, the catalase test, the latex-slide agglutination test for clumping factor and protein A (PS test, Eiken Chemistry Co., Ltd., Japan), and the tube coagulase test (18, 19). Coagulase typing (19) was carried out using a coagulase typing kit (Denka Seiken, Niigata, Japan). The strains were stored in 10% skim milk at −85 C until use, and maintained on Trypticase-Soy agar (TSA) with 5% sheep blood (BD Japan, Tokyo).

Antimicrobial susceptibility testing. Susceptibility testing was performed on the Walkaway System SI using the Pos Combo Panel Type 41J (Dade Behring, Inc., West Sacramento, Calif., U.S.A.). The antimicrobial agents tested were oxacillin (OXA), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), levofloxacin (LVX), vancomycin (VAN), teicoplanin (TEC) and trimethoprim/sulfamethoxazole (SXT). Minimal inhibitory concentrations (MICs) were interpreted as sensitive or resistant based on National Committee for Clinical Laboratory Standards (NCCLS) guidelines (27). With respect to CO-MRSA strains, supplemental MIC testing for OXA and methicillin (MET) using Etest (AB Biodisk, Solna, Sweden) was performed. Resistance to spectinomycin (SPT) and tetracycline (TET) was determined by plating strains on TSA containing 500 µg/ml of SPT, and 40 µg/ml of TET as previously described (19). In addition to resistance to OXA, multi-drug resistance (MDR) was defined as resistance to 4 or more different antimicrobials among the 9 drugs of GEN, ERY, CLI, LVX, VAN, TEC, SXT, SPT, and TET.

Pulsed-field gel electrophoresis analysis. Preparation of chromosomal DNAs and pulsed-field gel electrophoresis (PFGE) analysis was done as described previously (7). PFGE profiles were visually analyzed in addition to analysis using the Lane Multi Screener software version 3.0 (ATTO Co., Tokyo). The PFGE patterns were interpreted according to the criteria of Tenover et al. (40). Isolates showing six or fewer fragment differences were considered to be subtypes of a pulse type (40).

Detection of enterotoxins, exfoliative toxins, TSST-1 and the Panton-Valentine leukocidin (PVL) genes. Crude DNA extraction for PCR analysis was performed as previously described (19). Genes coding for staphylococcal enterotoxins SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), and SEE (*see*); exfoliative toxin A, B (ETA;

eta, ETB; *etb*); and TSST-1 (*tst*) were detected using a multiplex PCR system as previously described (3, 19). The PVL gene was detected by PCR assay described by Lina et al. (23).

*Typing of SCC*mec. SCC*mec* typing was performed by amplification of regions within SCC*mec*, the *ccr* region (three classes of *ccr*), and the *mec* region (IS*1272*, *mecI-R1*, *mecA*) as described by Ito et al. (16, 17) and Ma et al. (24). CO-MRSA strains of SCC*mec* type II or IV were subtyped (IIa, IIb) (IVa, IVb, IVc) as reported previously (T. Ito, X.X. Ma, Y. Kondo, P. Changtrakool, S. Traklsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. Song, and K. Hiramatsu, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 115, 2004). Control strains of each SCC*mec* type were provided by Drs. Robert S. Daum and T. Ito (9, 16, 17).

Multi-locus sequence typing (MLST). MLST was performed as described elsewhere (11). The allelic profiles of MRSA isolates were assigned on the basis of their MLST type using the eBURST program (13) (http://eburst.mlst.net).

Statistical analysis. To compare differences between groups, Student's *t* test was used for continuous variables, while Fisher's exact test was used for dichotomous variables. All analyses were two-tailed and *P* values of less than 0.05 were considered significant. Statistical analysis was performed with StatView version 5.0 software (Abacus Concepts, Inc., Berkeley, Calif., U.S.A.).

Results

Patient Characteristics

Of the 200 patients with MRSA strains during the study, 28 cases were excluded on the basis of duplicate isolates or misclassification of methicillin-susceptible strains. A total of 172 cases, including 98 inpatients with HA-MRSA, 61 outpatients with HA-MRSA, and 13 patients with CO-MRSA, were subjected to further analysis. Table 1 describes the characteristics of patients and specimens in the study. The age distribution of both inpatients and outpatients with HA-MRSA strains was significantly higher than that of patients with CO-MRSA. There were no patients over 60 years old with CO-MRSA. Sixty-two percent (8 of 13) of CO-MRSA strains were isolated from skin sources while sputum (29 of 98: 30%) was the major source of HA-MRSA strains in inpatients. Otorrhea (19 of 61: 31.1%) was the major source of specimens for outpatients. Forty-six percent (6 of 13) of patients with CO-MRSA were seen by the dermatology department, while inpatients with HA-MRSA were followed by the surgery (43 of 98: 44%) and the medicine (26 of 98: 27%) departments. Outpatients with HA-MRSA strains were seen mainly in the otolaryngology department (21 of 61: 34.4%).

Resistant Profile

Table 2 demonstrates the 9 resistant profiles, excluding β-lactams, of HA-MRSA and CO-MRSA strains. Most HA-MRSA strains from inpatients and outpatients were uniformly MDR. The resistant rate of CO-MRSA strains for ERY, CLI, TET, LVX, and SPT was significantly lower than that of HA-MRSA strains. However, GEN resistance was seen more frequently in CO-MRSA than in HA-MRSA strains. No resistance to SXT, VAN and TEC was seen in the CO-MRSA and the HA-MRSA strains recovered during the study.

Genotypes and Phenotypes of MRSA Strains

Table 3 shows genotypic and phenotypic characteristics of 172 MRSA strains evaluated in the prospective study conducted at TWMUH. The PFGE patterns of the MRSA strains revealed one major clone, clone A (145 of 172; 84%) with 51 subtypes (A1 to A51), three minor clones, clone B (8 of 172; 5%) with 7 subtypes (B1 to B7), clone C (5 of 172; 3%) with 4 subtypes (C1 to C4), clone D (3 of 172; 2%) with 3 subtypes (D1 to D3), and 11 unique clones (E to O). Most clone A strains were SCC*mec* type II (141 of 145; 97%), possessed *tst* or *sec* (136 of 145; 94%), coagulase type II (138 of 145; 95%), and MDR (143 of 145; 99%). Clone A was the most prevalent clone in HA-MRSA from both inpatients (89 of 98; 91%) as well as outpatients (53 of 61; 87%), and was significantly less frequently seen in CO-MRSA strains (3 of 13; 23%). Clone B strains were only isolated from HA-MRSA in outpatients or from CO-MRSA, belonged to coagulase type I and exhibited no MDR. SCC*mec* type IV (6 of 8; 75%) or *etb* (5 of 8; 63%) was frequently seen in clone B strains, although two strains belonged to SCC*mec* type II. Clone C strains belonged to coagulase type II, were MDR possessed *tst* and *sec* similar to clone A although the SCC*mec* type was non-typeable in two strains. Clone D strains were coagulase type III and were not MDR, but interestingly two of the three strains possessed *tst* and *sec*. This clone was not isolated from HA-MRSA in inpatients. All of the SCC*mec* type IV isolates including three HA-MRSA strains exhibited no MDR. Among inpatients and outpatients, Table 4 summarizes the correlation of genotypic or phenotypic characteristics between HA-MRSA and CO-MRSA strains. SCC*mec* type IV was more frequently distributed in CO-MRSA strains (9 of 13; 69%) than in HA-MRSA strains among inpatients (1 of 98; 1%) and in

Table 1. Demographic characteristics of patients and sources of specimens

Characteristics	Age distribution or number (%) of patients with HA-MRSA isolates from Inpatients (n=98)	P value[a]	P value[b]	Outpatients (n=61)	P value[a]	Total (n=159)	P value[a]	CO-MRSA isolates (n=13)
Age								
Median, year	56			58		56		19
Range, year	0–92			0–88		0–92		1–53
Mean±SD, year	47.0±28.8	0.002	NS	48.7±24.4	0.0002	47.6±27.1	0.002	20.8±18.9
0–5	18 (18)	NS[c]	NS	6 (10)	NS	24 (15)	NS	4 (31)
6–12	1 (1)	NS	NS	1 (2)	NS	2 (1)	NS	1 (8)
13–17	1 (1)	NS	NS	1 (2)	NS	2 (1)	NS	1 (8)
18–59	33 (34)	NS	NS	25 (41)	NS	58 (37)	NS	7 (54)
≥60	45 (46)	0.004	NS	28 (46)	0.005	73 (46)	0.003	0 (0)
Gender								
Male	63 (64)	NS	NS	33 (54)	NS	96 (60)	NS	9 (69)
Source of specimen								
Skin, soft tissue	16 (16)	0.01	NS	16 (26)	0.02	32 (20)	0.02	8 (62)
Discharge of eyes	6 (6)	NS	NS	3 (5)	NS	9 (6)	NS	2 (15)
Otorrhea	2 (2)	NS	<0.0001	19 (31)	NS	21 (13)	NS	1 (8)
Nose	10 (10)	NS	NS	8 (13)	NS	18 (11)	NS	0 (0)
Oropharynx	11 (11)	NS	NS	3 (5)	NS	14 (9)	NS	0 (0)
Tonsil	0 (0)	0.01	NS	0 (0)	0.03	0 (0)	0.0003	2 (15)
Sputum, BAL[d] fluid	29 (30)	0.02	<0.0001	3 (5)	NS	32 (20)	NS	0 (0)
Pleural fluid/ascites	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	0 (0)
Wound pus, deep	13 (13)	NS	NS	6 (10)	NS	19 (12)	NS	0 (0)
Blood	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	0 (0)
Urine	8 (8)	NS	NS	2 (3)	NS	10 (6)	NS	0 (0)
Feces	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	0 (0)
Breast milk	0 (0)	NS	NS	1 (2)	NS	1 (1)	NS	0 (0)
Clinic								
Medicine	26 (27)	0.04	NS	13 (21)	NS	39 (25)	0.04	0 (0)
Surgery	43 (44)	0.01	0.01	14 (23)	NS	57 (36)	NS	1 (8)
Pediatrics	9 (9)	NS	NS	8 (13)	NS	17 (11)	NS	1 (8)
Emergency	5 (5)	NS	NS	0 (0)	NS	5 (3)	NS	0 (0)
Otolaryngology	5 (5)	NS	<0.0001	21 (34)	NS	26 (16)	NS	3 (23)
Ophthalmology	4 (4)	NS	NS	3 (5)	NS	7 (4)	NS	2 (15)
Dermatology	2 (2)	<0.0001	NS	2 (3)	0.0002	4 (3)	<0.0001	6 (46)
Gynecology and Obstetrics	2 (2)	NS	NS	0 (0)	NS	2 (1)	NS	0 (0)
Psychiatry	2 (2)	NS	NS	0 (0)	NS	2 (1)	NS	0 (0)

[a] P value, vs. CO-MRSA.
[b] P value, vs. HA-MRSA from outpatients.
[c] NS, not significant.
[d] BAL, broncho-alveolar lavage.

outpatients (2 of 61; 3%). No SCC*mec* type III and type V strains were seen in any MRSA strains. Toxin gene profiles revealed that *sec* and *tst* was more predominant in HA-MRSA strains from inpatients (90 of 98; 92%, 88 of 98; 90%) and outpatients (49 of 61; 80%, 52 of 61; 85%) than in CO-MRSA strains (4 of 13; 31%, 5 of 13; 39%). In contrast, the presence of *etb* was more likely in CO-MRSA strains (4 of 13; 31%) than in HA-MRSA (inpatient 0 of 98; 0%, outpatient 1 of 61; 2%, total 1 of 159; 1%). No *pvl* genes were found in any MRSA. Coagulase typing showed that most HA-MRSA strains belonged to type II (inpatient 94 of 98; 96%, outpatient 49 of 61: 80.3%, total 143 of 159: 89.9%), and CO-MRSA were distributed into 5 type I, 4 type II, 2 type III and type VII, and non-typeable strains. PFGE clone A-coagulase type II-*tst*

Table 2. Resistant profiles of MRSA strains

Antimicrobial agent	Number (%) resistant							
	HA-MRSA isolates from							CO-MRSA isolates
	Inpatients (n=98)			Outpatients (n=61)		Total (n=159)		
		P value[a]	P value[b]		P value[a]		P value[a]	(n=13)
GEN	40 (41)	0.02	NS	24 (39)	0.02	64 (40)	0.02	10 (77)
ERY	97 (99)	0.0006	NS	57 (93)	0.03	154 (97)	0.002	9 (69)
CLI	88 (90)	<0.0001	NS	52 (85)	0.0002	140 (88)	<0.0001	4 (31)
TET	73 (74)	0.003	NS	48 (79)	0.001	121 (76)	0.001	4 (31)
LVX	91 (93)	<0.0001	NS	56 (92)	<0.0001	147 (92)	<0.0001	4 (31)
SPT	97 (99)	<0.0001	NS	58 (95)	<0.0001	155 (97)	<0.0001	5 (38)
SXT	0 (0)	NS[c]	NS	0 (0)	NS	0 (0)	NS	0 (0)
VAN	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
TEC	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)

[a] P value, vs. CO-MRSA.
[b] P value, vs. HA-MRSA from outpatients.
[c] NS, not significant.

and *sec* positive-coagulase type II-MDR strains were predominant in HA-MRSA from inpatients (81 of 98; 83%) and outpatients (43 of 61; 71%), as compared in CO-MRSA (1 of 13; 8%).

Properties of CO-MRSA Isolates and the Clinical Backgrounds

Table 5 summarizes genetic and phenotypic properties of CO-MRSA isolates as well as the clinical backgrounds. Clinically, 8 of 13 (62%) patients with CO-MRSA were diagnosed as skin infections including 3 superficial skin infections and 5 impetigo cases. No mortality was associated with infections due to CO-MRSA. Thirteen CO-MRSA strains were divided into 8 resistant profiles, 5 coagulase types, 5 toxin patterns, 2 SCC*mec* types with 3 subtypes, 12 different PFGE patterns (Fig. 1) including 3 clones A, 5 clones B, 2 clones D and 3 unique clones, and 7 sequence types with 4 clonal complexes (CC) types in MLST. Three strains belonged to MLST ST5-SCC*mec* type II, subtype IIa-PFGE clone A, coagulase type II and were MDR. These strains were closely related to major clones of HA-MRSA strains which shared SCC*mec* type II, coagulase type II, and MDR. As shown in Fig. 1, the PFGE patterns of 3 CO-MRSA strains were similar to that of HA-MRSA strain TWCC8395 (PFGE A11). These PFGE type A strains and TWCC8395 also belonged to MLST type ST5. The HA-MRSA strains with PFGE type A1 to A5 also belonged to ST5 (data not shown). TWCC8366 belonged to MLST ST5-SCC*mec* type IIa and coagulase type II which, while similar to TWCC8202, TWCC8254, and TWCC8290, was resistant only to ERY and SPT and showed a different PFGE pattern (unique L). All ST5-SCC*mec* IIa CO-MRSA strains showed high-level resistance to MET and OXA (MIC: >256 μg/ml). The remaining 9 strains belonged to SCC*mec* type IV and were moderately resistant to MET and OXA with a MIC range of 12–48 and 8–64 μg/ml, respectively. Five of nine SCC*mec* type IV CO-MRSA strains belonged to type IVa, but the remaining four strains did not amplify with primers for either a, b, or c types of the J1 region. Five SCC*mec* type IVa strains were characterized as demonstrating closely related PFGE patterns (clone B) and belonging to CC509 (3 ST91, ST89, ST379), and coagulase type I. These strains were resistant to only GEN or ERY except for one strain, TWCC8422, which was also resistant to SPT and possessed the *etb* genes except for one strain, TWCC8435. Three SCC*mec* type IVa strains possessing *etb* were isolated from impetigo cases. Four other SCC*mec* type IV, non-subtype IVa, IVb, IVc strains belonged to the following CC types: CC1 (ST1, ST81) and two CC8 (ST8). Two CC1 strains showed different resistant profiles (one MDR and the other no), PFGE types, and coagulase types consistent with independent clones. While, two CC8 (ST8) belonged to coagulase type III, exhibited closely related PFGE patterns, and were non-MDR. Both strains also had *tst* and *sec* genes and were isolated from impetigo patients, but did not have *etb* or *eta* genes. Three clone B (one ST91, two ST89-CC509, coagulase type I) and one clone D (ST8, CC8, coagulase III, SCC*mec* type IV) were also isolated from HA-MRSA, but 2 ST89-clone B (TWCC8081, TWCC8472) and clone D (TWCC8414) strains belonged to SCC*mec* type II, not type IV (data not shown).

Discussion

To our knowledge, this is the first prospective molec-

Table 3. Genotypic and phenotypic characterization of 172 MRSA strains

PFGE		SCC*mec*	Toxin gene	Coagulase type	MDR[a]	Number of strains				
						HA-MRSA from			CO-MRSA	Total
Type	Subtype					Inpatients	Outpatients	Total		
A	A1	II	*tst, sec*	I	Y		1	1		1
	A1	II	*tst*	II	Y				1	1
	A1	II	*tst, sec*	II	Y	16	9	25		25
	A1	II	*tst, sec*	V	Y		1	1		1
	A2	II	*tst, sec*	II	Y	6	2	8		8
	A2	II	*tst, seb, sec*	II	Y	3	1	4		4
	A2	II	*tst, seb, sec*	NT[b]	Y	1		1		1
	A3	II	*tst, sec*	II	Y	6	5	11		11
	A4	II	*tst, sec*	II	Y	4	2	6	1	7
	A4	II	*tst, sec*	V	Y		1	1		1
	A5	II	*sec*	II	Y	1		1		1
	A5	II	*tst, sec*	II	Y	5	1	6		6
	A5	II	*tst, sec*	IV	Y	1		1		1
	A6	II	*tst, sec*	II	Y	4	1	5		5
	A6	II	*tst, sec*	V	Y		1	1		1
	A7	II	*tst, sec*	II	Y	3		3		3
	A7	II	*tst, seb, sec*	II	Y		1	1		1
	A8	II	*tst, sec*	II	Y	4		4		4
	A9	II	*tst, sec*	II	Y	2	2	4		4
	A10	II	*tst*	II	Y		1	1		1
	A10	II	*tst, sec*	II	Y	1	1	2		2
	A10	NT	*tst*	II	Y		1	1		1
	A11	II	*tst, sec*	II	Y	2	1	3		3
	A12	II	*tst, sec*	II	Y	3		3		3
	A13	II	*tst, seb, sec*	II	Y	3		3		3
	A14	II	none	II	Y	2	1	3		3
	A15	II	*tst, sec*	II	Y	1	1	2		2
	A16	II	none	II	Y	1		1		1
	A16	II	*tst, sec*	II	Y	1		1		1
	A17	II	*tst, sea, sec*	II	Y	2		2		2
	A18	II	*tst, sec*	II	Y	1	1	2		2
	A19	II	*tst, sec*	II	Y	1	1	2		2
	A20	II	*tst, sec*	II	Y	1	1	2		2
	A21	II	*tst, sec*	II	Y	1		1		1
	A22	II	*tst, sec*	II	Y	1		1		1
	A23	II	*tst, sec*	II	Y	1		1		1
	A24	II	*tst, sec*	II	Y	1		1		1
	A25	II	*tst, seb, sec*	II	Y	1		1		1
	A26	II	none	II	Y	1		1		1
	A27	II	none	II	Y		1	1		1
	A28	II	*tst, sec*	II	Y		1	1		1
	A29	II	none	II	Y		1	1		1
	A30	II	*tst, sec*	II	Y		1	1		1
	A31	II	*tst, sec*	II	Y	1		1		1
	A32	II	*tst, sec*	II	Y		1	1		1
	A33	II	*tst, sec*	II	Y		1	1		1
	A34	II	*tst, sec*	II	Y		1	1		1
	A35	II	*tst, sec*	II	Y	1		1		1
	A36	II	*tst, sec*	II	Y		1	1		1
	A37	II	*tst, sec*	II	Y		1	1		1
	A38	II	*tst, sec*	II	Y	1		1		1
	A39	II	none	II	Y				1	1
	A40	II	*tst, sec*	II	Y		1	1		1
	A41	I	*seb*	II	N	1		1		1

Table 3. continued

PFGE Type	PFGE Subtype	SCC*mec*	Toxin gene	Coagulase type	MDR[a]	HA-MRSA from Inpatients	HA-MRSA from Outpatients	HA-MRSA Total	CO-MRSA	Total
A	A42	II	*tst, sec*	II	Y	1		1		1
	A43	II	*tst, sec*	II	Y		1	1		1
	A44	II	*tst, sec*	II	Y		1	1		1
	A45	NT[b]	*tst, sec*	V	N		1	1		1
	A46	NT	*tst, sec*	II	Y		1	1		1
	A47	II	*tst, sec*	II	Y	1		1		1
	A48	II	*tst, sec*	II	Y		1	1		1
	A49	II	*tst, sec*	II	Y	1		1		1
	A50	II	*tst, sec*	II	Y	1		1		1
	A51	II	*tst, sec*	II	Y		1	1		1
	Total					89	53	142	3	145
B	B1	IV	*etb*	I	N				1	1
	B2	IV	*etb*	I	N				1	1
	B2	IV	none	I	N				1	1
	B3	IV	*etb*	I	N				1	1
	B4	IV	*etb*	I	N				1	1
	B5	II	*etb*	I	N		1	1		1
	B6	II	none	I	N		1	1		1
	B7	IV	none	I	N		1	1		1
	Total					0	3	3	5	8
C	C1	II	*tst, sec*	II	Y		1	1		1
	C1	NT	*tst, sec*	II	Y	1		1		1
	C2	NT	*tst, sec*	II	Y	1		1		1
	C3	II	*tst, seb, sec*	II	Y	1		1		1
	C4	II	*tst, sec*	II	Y	1		1		1
	Total					4	1	5	0	5
D	D1	IV	*tst, sec*	III	N				1	1
	D2	II	none	III	N		1	1		1
	D3	IV	*tst, sec*	III	N				1	1
	Total					0	1	1	2	3
Unique										
E		I	none	III	Y	1		1		1
F		II	*tst, sec*	II	Y	1		1		1
G		IV	none	III	N		1	1		1
H		IV	*sea*	VII	N				1	1
I		IV	none	NT	Y				1	1
J		II	none	II	Y	1		1		1
K		II	none	III	Y		1	1		1
L		II	*tst, sec*	II	N				1	1
M		I	none	II	N	1		1		1
N		II	*tst, sec*	II	Y		1	1		1
O		IV	*sec*	VII	N	1		1		1
	Total					5	3	8	3	11
Total						98	61	159	13	172

[a] MDR, multi-drug resistance, Y: yes, N: no.
[b] NT, non-typeable.

ular epidemiological study of CO-MRSA and HA-MRSA in a Japanese hospital. Overall, patients with CO-MRSA were significantly younger than those with HA-MRSA and seen mainly in the dermatology service. A previous study reported results similar to our study with respect to age distribution, and clinical features in patients with CO-MRSA infection (4–6, 10, 26, 32, 34, 43). Our study found CO-MRSA strains to be more susceptible to antimicrobials other than β-lactam classes except GEN. Furthermore, SCC*mec* type IV, recently described in other CO-MRSA isolates, predominated in our CO-MRSA strains. These bacterial

Table 4. Comparison of genotype and phenotype distribution in HA-MRSA and CO-MRSA strains

Characteristics	Number of positive (%) in							
	HA-MRSA isolates from							CO-MRSA isolates
	Inpatients (n=98)			Outpatients (n=61)		Total (n=159)		
		P value[a]	P value[b]		P value[a]		P value[a]	(n=13)
SCC*mec*								
I	3 (3)	NS[c]	NS	0 (0)	NS	3 (2)	NS	0 (0)
II	91 (93)	<0.0001	NS	55 (90)	<0.0001	146 (92)	<0.0001	4 (31)
III	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
IV	1 (1)	<0.0001	NS	2 (3)	<0.0001	3 (2)	<0.0001	9 (69)
V	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
NT[d]	3 (3)	NS	NS	4 (7)	NS	7 (4)	NS	0 (0)
PFGE								
Clone A	89 (91)	<0.0001	NS	53 (87)	<0.0001	142 (89)	<0.0001	3 (23)
Clone B	0 (0)	<0.0001	NS	3 (5)	0.003	3 (2)	<0.0001	5 (39)
Clone C	4 (4)	NS	NS	1 (2)	NS	5 (3)	NS	0 (0)
Clone D	0 (0)	0.013	NS	1 (2)	NS	1 (1)	0.02	2 (15)
Unique clone	5 (5)	NS	NS	3 (5)	NS	8 (5)	0.04	3 (23)
Toxin gene								
eta	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
etb	0 (0)	0.0001	NS	1 (2)	0.003	1 (1)	<0.0001	4 (31)
sea	2 (2)	NS	NS	0 (0)	NS	2 (1)	NS	1 (8)
seb	10 (10)	NS	NS	2 (3)	NS	12 (8)	NS	0 (0)
sec	90 (92)	<0.0001	0.05	49 (80)	0.0009	139 (87)	<0.0001	4 (31)
sed	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
see	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
tst	88 (90)	<0.0001	NS	52 (85)	0.001	140 (88)	0.0001	5 (39)
pvl	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
none	7 (7)	NS	NS	8 (13)	NS	15 (9)	NS	3 (23)
Coagulase type								
I	0 (0)	<0.0001	0.02	4 (7)	0.007	4 (3)	0.0001	5 (39)
II	94 (96)	<0.0001	0.02	49 (80)	0.0009	143 (90)	<0.0001	4 (31)
III	1 (1)	0.04	NS	3 (5)	NS	4 (3)	NS	2 (15)
IV	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	0 (0)
V	0 (0)	NS	0.008	5 (8)	NS	5 (3)	NS	0 (0)
VI	0 (0)	NS	NS	0 (0)	NS	0 (0)	NS	0 (0)
VII	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	1 (8)
NT	1 (1)	NS	NS	0 (0)	NS	1 (1)	NS	1 (8)
Multi-drug resistance (MDR)	95 (97)	<0.0001	NS	55 (90)	<0.0001	150 (94)	<0.0001	4 (31)
Clone A: SCC*mec* II: *sec*+*tst*: coagulase II: MDR strains	81 (83)	<0.0001	NS	43 (71)	<0.0001	124 (78)	<0.0001	1 (8)

[a] P value, vs. CO-MRSA.
[b] P value, vs. HA-MRSA from outpatients.
[c] NS, not significant.
[d] NT, non-typeable.

features were also similar to previous reports (4–6, 22, 26, 28, 30, 32, 42, 43). However, in our study, we found no CO-MRSA strains harboring *pvl* genes which have been reported predominantly in CO-MRSA strains from skin or soft tissue infections as well as, necrotizing pneumonia outside of Japan (10, 22, 26, 30, 42, 43). Vandenesch et al. reported that all 117 CO-MRSA isolates collected from countries in three continents including the United States, Australia, France, and Switzerland carried *pvl* genes and belonged to SCC*mec* type IV (42). It has been suggested that the *pvl* genes may be a good marker for detecting CO-MRSA, but its absence in some CO-MRSA isolates from Australia, the United States and Switzerland (22, 28, 30).

The genetic diversity of CO-MRSA strains has been reported (4, 6, 8, 28, 30, 42). As suggested by recent studies, CO-MRSA strains are characterized by multiple genetic lineages (4, 6, 8, 28, 30, 42). In fact, our nine SCC*mec* type IV CO-MRSA strains contained 6 ST and 3 CC types.

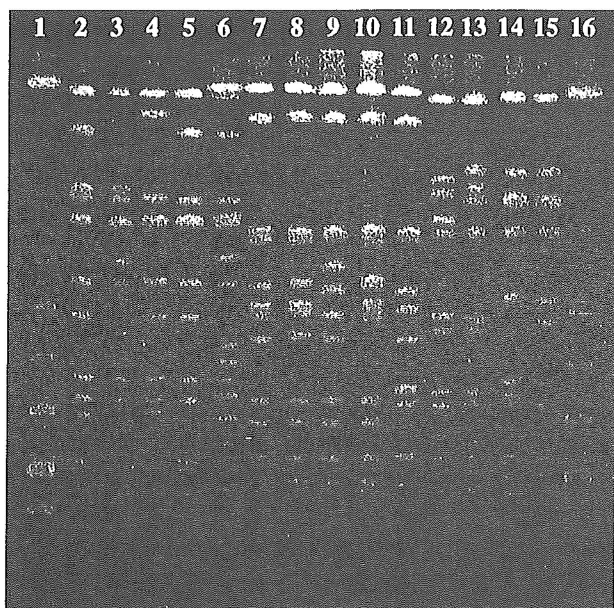

Fig. 1. PFGE patterns of 13 CO-MRSA isolates and one typical HA-MRSA strain. Low molecular-weight λDNA ladder markers are shown in lane 1 and 16. Lanes 2–15 display results obtained with strains: lane 2, TWCC8290 (C, type A4), lane 3, TWCC8254 (C, type A39), lane 4, TWCC8202 (C, type A1), lane 5, TWCC8395 (H, type A11, ST5, CC5), lane 6, TWCC8336 (C, type L), lane 7, TWCC8422 (C, type B2), lane 8, TWCC8435 (C, type B2), lane 9, TWCC8454 (C, type B3), lane 10, TWCC8453 (C, type B4), lane 11, TWCC8288 (C, type B1), lane 12, TWCC8149 (C, type H), lane 13, TWCC8442 (C, type I), lane 14, TWCC8403 (C, type D1), lane 15, TWCC8441 (C, type D3). Letters in parentheses above show: CO-MRSA (C), HA-MRSA (H) and each PFGE type.

The PFGE clone B-coagulase type I lineage (five strains) showed archetypal microbiological features such as low level resistance to OXA, non-MDR, and was predominately isolated from skin infections. The MLST types of these strains belonged to CC509 and ST89, 91, 379 which are uncommonly seen in nosocomial isolates and have very rarely been reported as community-onset isolates. To date, we found only one ST89 and one ST91 MRSA strain from Japan (1, http://www.mlst.net/BURST/burst.htm); however the clinical history of the patients could not be clarified. Eighty percent (four of five) of the strains in this lineage harbored *etb* genes. ETB is one of the virulence factors associated with impetigo and staphylococcal scalded skin syndrome (45). Yamaguchi et al. described 6 similar *etb*-positive coagulase type I MRSA isolated from bullous impetigo patients in the western region of the main island of Japan (45). Moreover, these strains showed similar resistant profiles to our coagulase type I-CC509 isolates including low-level resistance to OXA, susceptibility to minocycline, and resistance to GEN.

Table 5. Properties of CO-MRSA and genetically-related isolates and their clinical backgrounds

Isolate	Background of patients				MIC (μg/ml) of		Resistant profile	Coagulase	Toxin	SCC*mec*	PFGE	MLST analysis		
	Age	Sex	Infectious disease	Source	MET	OXA		type	gene	type	type	Allelic profile	ST	CC
TWCC8202	53	M	Burn skin infection	Skin	>256	>256	GEN, ERY, CLI, LVX, SPT, TET	II	*tst*	IIa	A1	1-4-1-4-12-1-10	5	5
TWCC8254	23	M	Superficial skin infection	Skin	>256	>256	ERY, CLI, LVX, SPT, TET	II	none	IIa	A39	1-4-1-4-12-1-10	5	5
TWCC8290	52	M	Superficial skin infection	Skin	>256	>256	ERY, CLI, LVX, SPT, TET	II	*tst, sec*	IIa	A4	1-4-1-4-12-1-10	5	5
TWCC8336	23	F	Acute tonsillitis	Tonsil	>256	>256	ERY, SPT	II	*tst, sec*	IIa	L	1-4-1-4-12-1-10	5	5
TWCC8149	19	F	Conjunctivitis	Eye	16	32	GEN	VII	*sea*	IV(non a,b,c)	H	1-1-1-1-1-1-1	1	1
TWCC8442	47	M	Chronic otitis media	Otorrhea	32	16	GEN, ERY, CLI, LVX, SPT	NT[a]	none	IV(non a,b,c)	I	1-1-1-9-1-1-1	81	1
TWCC8403	22	M	Impetigo	Skin	48	64	GEN, TET	III	*tst, sec*	IV(non a,b,c)	D1	3-3-1-1-4-4-3	8	8
TWCC8441	3	M	Impetigo	Skin	32	32	GEN	III	*tst, sec*	IV(non a,b,c)	D3	3-3-1-1-4-4-3	8	8
TWCC8288	6	M	Impetigo	Skin	12	8	GEN, ERY	I	*etb*	IVa	B1	1-26-28-18-18-33-50	89	509
TWCC8422	1	M	Impetigo	Skin	32	16	GEN, ERY, SPT	I	*etb*	IVa	B2	1-26-28-18-59-54-50	379	509
TWCC8435	13	M	Acute rhinitis-tonsillitis	Tonsil	32	24	GEN	I	none	IVa	B2	1-26-28-18-18-54-50	91	509
TWCC8454	4	F	Conjunctivitis	Eye	16	24	GEN, ERY	I	*etb*	IVa	B3	1-26-28-18-18-54-50	91	509
TWCC8453	4	F	Impetigo	Skin	16	16	GEN, ERY	I	*etb*	IVa	B4	1-26-28-18-18-54-50	91	509

[a] NT, non-typeable.

Although we did not compare PFGE profiles directly or MLST analysis between our strains and Yamaguchi's strains, both lineages share similar microbiological features. These results indicate that most likely the dissemination of such *etb*-positive MRSA strains into the community have occurred in Japan. Yamaguchi et al. also reported that 9 coagulase type I *etb*-positive strains in this lineage, including 6 MRSA and 3 methicillin-susceptible *S. aureus* (MSSA), were grouped in a single cluster by PFGE analysis (45).

Although the origin of CO-MRSA strains remains speculative, they may have recently emerged by horizontal transfer of type IV SCC*mec* into a methicillin-susceptible lineage (10, 12, 17, 31). As it is the smallest of the 4 known SCC*mec* elements (21–24 kb in size), type IV SCC*mec* is probably more mobile than other classes of SCC*mec* (10, 12, 17, 31). *In vitro*, the growth rates of most CO-MRSA isolates are significantly faster than those of HA-MRSA, and comparable to that of MSSA (1, 21, 30). It can be speculated that such a selective advantage becomes a driving force in the dissemination of this CO-MRSA lineage benefits become driving forces to success this CO-MRSA lineage in the community of Japan. Interestingly, 3 other clone B strains were isolated from HA-MRSA. Two of the three clone B-ST89-CC509 HA-MRSA strains showed SCC*mec* type II, instead of type IV. One clone D HA-MRSA strain also belonged to SCC*mec* type II. There were some reports that the same PFGE clone strains shared SCC*mec* type II and type IV (4, 35).

The other four SCC*mec* type IV strains contained two lineages (CC1 and ST1 or ST81, CC8 and ST8). The CC1 strains have the same MLST allelic profile and coagulase type as that of the *S. aureus* strain which is the proposed ancestor of MW2, a CO-MRSA strain responsible for the deaths of four children in the United States (30). Furthermore, CC1 CO-MRSA strains have been reported in Australia with or without *pvl* genes (28, 30). CC8 CO-MRSA clones also have already become widely disseminated with community-onset infection in the United States and Australia (4, 6, 8, 28, 30, 42). Okuma et al. reported that two ST8 CO-MRSA strains from the United States belonged to coagulase type III (30) as was the case of TWCC8403 and TWCC8441. These results indicate that both CC1 and CC8 CO-MRSA strains may be particularly successful lineages. Type IV SCC*mec* has been classified into three subtypes based on sequence difference in the J1 region (17, 24, 30). Most CC1 or CC8 CO-MRSA strains belong to SCC*mec* type IVa, but subtypes of our strains could not be determined by PCR amplification of the J1 region (17, 24, 30). Similar untypeable SCC*mec* type IV CO-MRSA strains have been reported (28, 35). Moreover, type IV SCC*mec* is widely distributed among MRSA or coagulase-negative staphylococci (CNS), with many potential reservoirs of this gene. SCC*mec* type IV may transfer from CNS to MSSA or vice versa (14, 44). These results indicate that type IV SCC*mec* has more genetic diversity than the other four types.

The remaining four CO-MRSA strains resemble HA-MRSA isolates in having characteristics such as MDR, coagulase type II, SCC*mec* type II, and MLST CC5 and ST5, and to some extent, could be easily distinguished from the SCC*mec* type IV CO-MRSA strains. This lineage is the same as a HA-MRSA New York/Japan clone that is widely disseminated in hospitals in the United States and Japan (1, 2, 4, 18–20, 26). The strict definition of "community-acquired," "community-associated" or "community-onset" infection is still controversial (34). Some previous reports have described CO-MRSA as the result of migration of HA-MRSA overflow from hospital environments to the community (6, 39). In this study, we differentiated HA-MRSA strains on the basis of inpatient and outpatient-origin. Sixty-one out of seventy-four MRSA strains from outpatients belonged to HA-MRSA. Surprisingly, HA-MRSA strains from outpatients were most frequently isolated from otorrhea such as chronic otitis media or externa. One CO-MRSA strain TWCC8442 was also isolated from otorrhea of a patient with chronic otitis media. Some authors have reported that MRSA infections appear to be common in chronic otitis media (15, 36). Although the actual origins and transmission routes of CO-MRSA have not been still elucidated, our data suggest that chronic ear infections might be one of the origins of HA-MRSA into the community. Conversely, in our study three strains of HA-MRSA belonged to SCC*mec* type IV. These three strains (TWCC8331, 8409, 8479) also showed no MDR as in the case of CO-MRSA. These results suggest that some CO-MRSA strains may spread via nosocomial transmission. There have been several reports of CO-MRSA outbreaks originating from a hospital transmission (29, 33). While further studies are required, one should be aware of the epidemiological trends of CO-MRSA and HA-MRSA in the community.

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Medical Tribune 2004年４月１日号特別企画より転載

マイコプラズマ肺炎の新知見とマクロライドの可能性

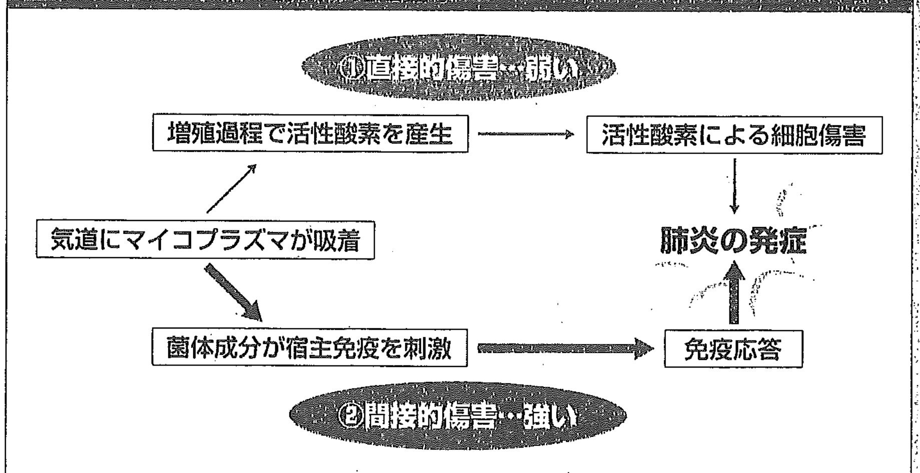
小児や若年者に多いマイコプラズマ肺炎の病態は，宿主の免疫機構により形成されることが明らかになりつつある。そこで，マイコプラズマ肺炎の病態形成に重要な役割を果たしているとされる炎症性サイトカインの関与や，14員環マクロライド系薬の抗炎症作用による治療の有効性などについて，マイコプラズマ感染症の病態を研究している札幌鉄道病院小児科医長の成田光生氏に聞いた。

コメンテーター
札幌鉄道病院小児科医長
成田 光生 氏

マイコプラズマ肺炎の発症メカニズム

近年マイコプラズマ肺炎の病態形成において，直接的傷害に加え宿主の免疫応答による間接的傷害が大きく関与することが明らかになり注目されている。

体内の気道に侵入したマイコプラズマは，気道上皮細胞の線毛間に吸着し，その部位で宿主の栄養分を吸収しながら増殖を繰り返す。そのプロセスで活性酸素を放出し，上皮細胞の傷害を引き起こす(直接的傷害)。また，マイコプラズマの菌体成分が宿主免疫を刺激して，炎症反応を引き起こし，細胞傷害を増強させる(間接的傷害)(図1)。



重要な役割果たすIL-18，8

成田氏は，この免疫機構による細胞傷害の作用機序を明らかにするため，10種類近くのサイトカインの関与について検討した。それによると，マイコプラズマ肺炎の発症機序として，ヘルパーT細胞 1型(Th1)にかかわるIL-18が重要な役割を果たしていることがわかった。IL-18はマクロファージなどからのサイトカインの産生を刺激していると考えられている。マイコプラズマ感染によりマクロファージから産生されたIL-18は，マクロファージに働いて炎症性サイトカインIL-8の分泌を促進させる。IL-8は好中球の遊走能を促進させ，病巣に好中球を中心とした炎症性細胞を集積させる。その結果，好中球などが産生する酵素などの傷害因子が気道上皮細胞を傷害してマイコプラズマ肺炎を成立させると考えられる。

免疫応答により病態が形成される

このように，マイコプラズマ肺炎の病態形成には，宿主の免疫系の深い関与が示唆される。成田氏はマイコプラズマ感染から治癒に至るまでの免疫系の関与について次のような仮説を立てる。

「感染が成立する病初期にはマイコプラズマが増殖するが，その後宿主の免疫応答が立ち上がってくるとマイコプラズマは排除され始め，その一方で肺炎の病像が形成される」(図2)