alleles associated with the wide spread. For analyzing differences in gene expression between bacterial strains, the proteomic approach is a powerful tool (4, 26). Thus, the comparative proteomic analysis of *B. pertussis* strains (type A and type B) was thought to be worth trying, and an attempt is now under way.

In conclusion, in the Japanese *B. pertussis* strains, the antigenic divergence between recently circulating strains and the vaccine strain has been observed since the mid-1990s, although reported pertussis-like and pertussis cases have decreased in number. In addition, the strains showed a correlation between the PFGE profile and the combination of *ptxS1/prn* alleles. Our findings strongly suggested that the antigenic divergence had no influence on the efficacy of pertussis vaccination in Japan. However, the reason for the appearance of the type B strain harboring nonvaccine *ptxS1A/prn2* has remained unclear. Continuous surveillance and further analyses are needed to determine the virulence of the type B strain.

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Two Distinct Patterns of Pleural Effusions Caused by *Mycoplasma pneumoniae* Infection

To the Editors:

We read with great interest the article by Wang et al.[1] The authors reported 5 pediatric patients with radiographically diagnosed necrotizing pneumonitis with pleural effusion caused by *Mycoplasma pneumoniae*, 2 of whom had persistent radiographic abnormalities lasting for more than several months. Complete resolution of chest roentgenograms was observed in the other 3 cases. Their presentation of the cases and its diagnostic implication were clinically valuable, but they did not mention the possible pathogenic mechanism of the disease. We suppose that the radiographic features that the authors presented for the cases with persistent abnormalities strongly suggest an organizing pneumonia with massive recruitment of neutrophils in histology.

In this context, we encountered similar pediatric cases of massive pleural effusion caused by *M. pneumoniae* infection with or without persistent radiographic abnormalities.[2] In the initial study including a total of 10 cases, 3 cases showed persistent radiographic abnormalities of fibrotic changes in the chest roentgenogram with the presence of *M. pneumoniae* genome detected by polymerase chain reaction in their pleural fluid samples. In the other 7 cases, radiographically abnormal findings were transient. In the subsequent report of 12 cases, we examined their pleural fluid samples for cytokines and found that interleukin (IL)-18 and IL-8 were significantly elevated in the pleural fluid samples obtained from the 4 patients with persistent radiographic abnormalities when compared with those without persistent abnormalities.[3,4]

On the basis of our findings, we strongly speculate that there are at least 2 distinct patterns of pleural effusions caused by *M. pneumoniae* infection: one type of effusion characterized by a transient chest disease, *M. pneumoniae* genome being undetectable, and with lower (but not normal) concentrations of IL-18 and IL-8 and the other type of effusion with a persistent chest disease, *M. pneumoniae* genome being detectable, and with significantly higher IL-18 and IL-8.

It is well-known that mycoplasmal cell membranes can elicit varieties of cytokine responses (for review, see Yang et al[5]), and some kinds of cytokines must play a significant role in producing the radiographic appearance of *M. pneumoniae* pneumonia not only for children but also for adults.[6,7] *M. pneumoniae* can induce IL-18 production through the activation of macrophages, and IL-8 production can be induced either indirectly through the function of IL-18 or directly through the function of Toll-like receptors which *M. pneumoniae* can also activate. In any case, it is reasonable to assume that IL-8 plays a pivotal role in constructing "necrotizing pneumonitis" or organizing pneumonia.

M. pneumoniae pneumonia is usually a benign, self-limited disease. Clinical samples which can be obtained from patients therefore are limited for use in clinical research. If one pays, in addition to routine laboratory testings, more attention to using newer analytical methodologies in analyzing pleural fluid samples obtained from patients with *M. pneumoniae* pneumonia, more information must be obtained for further understanding of the pathogenesis of *M. pneumoniae* infection.

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Reply:

The author proposed an interesting hypothesis about the pathophysiology of pleural effusion caused by *Mycoplasma pneumoniae*. It is biologically plausible that the author proposed that the presence of *M. pneumoniae* genome might elicit stronger immunologic reaction which subsequently leads to persistent lung damage. Yet the timing of sampling might bear some relevance to whether one could detect the genome in the pleural fluid. Before one can make a statement like this, a well-designed prospective study looking into the relationship of the presence of the genome versus the immunologic reaction and outcome is mandatory.

I do agree with the author that probably there are 2 types of pleural effusion associated with *M. pneumoniae* infection. On the basis of my clinical experience, I would advocate for more clinical and immunologic study in this area.

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Characterization and Molecular Analysis of Macrolide-Resistant *Mycoplasma pneumoniae* Clinical Isolates Obtained in Japan

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In recent years, *Mycoplasma pneumoniae* strains that are clinically resistant to macrolide antibiotics have occasionally been encountered in Japan. Of 76 strains of *M. pneumoniae* isolated in three different areas in Japan during 2000 to 2003, 13 strains were erythromycin (ERY) resistant. Of these 13 strains, 12 were highly ERY resistant (MIC, ≥256 μg/ml) and 1 was weakly resistant (MIC, 8 μg/ml). Nucleotide sequencing of domains II and V of 23S rRNA and ribosomal proteins L4 and L22, which are associated with ERY resistance, showed that 10 strains had an A-to-G transition at position 2063 (corresponding to 2058 in *Escherichia coli* numbering), 1 strain showed A-to-C transversion at position 2063, 1 strain showed an A-to-G transition at position 2064, and the weakly ERY-resistant strain showed C-to-G transversion at position 2617 (corresponding to 2611 in *E. coli* numbering) of domain V. Domain II and ribosomal proteins L4 and L22 were not involved in the ERY resistance of these clinical *M. pneumoniae* strains. In addition, by using our established restriction fragment length polymorphism technique to detect point mutations of PCR products for domain V of the 23S rRNA gene of *M. pneumoniae*, we found that 23 (24%) of 94 PCR-positive oral samples taken from children with respiratory infections showed A2063G mutation. These results suggest that ERY-resistant *M. pneumoniae* infection is not unusual in Japan.

Mycoplasma pneumoniae is a pathogen causing human respiratory infections such as atypical pneumonia, mainly in children and younger adults. In the chemotherapy of *M. pneumoniae* infection in children, erythromycin (ERY) and clarithromycin (CLR) among 14-membered macrolides and the 15-membered macrolide azithromycin (AZM) are usually considered the first-choice agents in Japan. Although there was no report on the isolation of ERY-resistant *M. pneumoniae* before 2000 in Japan, we found that ca. 20% of *M. pneumoniae* strains isolated from patients from 2000 to 2003 were ERY resistant. These results are consistent with pediatricians' impression that antibiotics such as ERY, CLR, and clindamycin (CLI) are not effective for some patients with *M. pneumoniae* infection.

It is well known that the macrolide-lincosamide-streptogramin B (MLS) antibiotics inhibit protein synthesis by binding to domain II and/or domain V of 23S rRNA (3, 26). Lucier et al. (10) and Okazaki et al. (17) found that an A-to-G transition or A-to-C transversion at position 2063 (corresponding to 2058 in *Escherichia coli* numbering) or 2064 of the 23S rRNA gene resulted in high resistance to macrolide antibiotics. No point mutation was found in domain II of 23S rRNA of the ERY-resistant *M. pneumoniae* strains used in the present study.

We report here the prevalence of macrolide-resistant *M. pneumoniae* infection in Japan. By using 13 ERY-resistant *M. pneumoniae* strains, we investigated the mechanisms of resistance to MLS antibiotics. Furthermore, we established restriction fragment length polymorphism (RFLP) techniques to detect point mutations in domain V of 23S rRNA of *M. pneumoniae* by using throat swabs or sputum samples.

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MATERIALS AND METHODS

Mycoplasmas. Three types of *M. pneumoniae* strains were used in the present study, i.e., ERY-resistant strains isolated from children infected with *M. pneumoniae* in Japan from 2000 to 2003, ERY-resistant strains induced with ERY in vitro, and three reference strains: M129, Mac, and FH. The ERY-resistant clinical isolates are listed in Table 1, with details regarding patient age, year of isolation, symptoms, and the administration of antibiotics. Most of the isolates

TABLE 1. Macrolide-resistant *M. pneumoniae* strains isolated from patients, along with patient information

Strain no.	Patient		Antimicrobial agent(s)[a]	
	Age (yr)	Symptoms and/or disease	First choice/effect	Second choice/effect
350	9	Pneumonia	CLI/−	CLR/+
374	3	Pneumonia	Unknown	Unknown
375	4.5	Pneumonia	Unknown	Unknown
376	12	Pneumonia	CLR/−	AZM/+
377	7	Fever and cough	AZM/+	
378	2	Fever and cough	Cefditoren pivoxil/−	AZM/+
379	9	Pneumonia	CLR/−	AZM/−
380	11	Pneumonia	CLR/−	Minocycline/+
381	11	Pneumonia	AZM/+	
382	7	Pneumonia	RKM/−	AZM/−
383	5	Bronchitis	Cefaclor/−	ERY/+
384	7	Pneumonia	Cefdinir, Fosfomycin/−	ERY/+
385	NI[b]	Pneumonia, pleurisy	CLR/+	

[a] −, No effect from antimicrobial agent; +, improvement of symptoms.
[b] NI, no information.

TABLE 2. Primers used for PCR amplification and sequencing of domains II and V of 23S rRNA and ribosomal proteins of L4 and L22 in *M. pneumoniae*

PCR and primer designation	Sequence (5′ to 3′)	Position[a]	Amplicon size (bp)
Domain II of 23S rRNA			
MN23SDIIF	AGTACCGTGAGGGAAAGGTG	491–510	816
MN23SDIIR	TCCCAAGCGTTACTCATGCC	1287–1306	
Domain V of 23S rRNA			
MN23SDVF	GCAGTGAAGAACGAGGGG	1758–1775	927
MN23SDVR	GTCCTCGCTTCGGTCCTCTCG	2664–2684	
Ribosomal protein L4			
MNL4F	AAAAGCAGCACCAGTTGTAG	1231–1250	722
MNL4R	GGTTAGAACTGGTTTTAGCA	1933–1952	
Ribosomal protein L22			
MNL22F	GTACATAACGGCAAGACCTT	3640–3659	627
MNL22R	GCAAGCCGTTGGAGTTTACT	4247–4266	
Nested PCR for 23S rRNA of 2063, 2064 region			
MN23SF1937	ACTATAACGGTCCTAAGGTA	1918–1937	210
MN23SR2128	ACCTATTCTCTACATGATAA	2108–2177	
Nested PCR for 23S rRNA of 2617 region			
MN23SF2577	TACGTGAGTTGGGTTCAAA	2577–2595	108
MN23SR2664	GTCCTCGCTTCGGTCCTCTCG	2664–2684	

[a] The positions of domain II and V of 23S rRNA are based on accession no. X68422 of the *M. pneumoniae* gene, and those of ribosomal proteins L4 and L22 are based on accession no. AE000061 of the *M. pneumoniae* M129 section 19 of 63 of the complete genome.

were obtained during the patient's first visit to the hospital, except in a few cases in which the isolates were obtained within a week after an initial treatment failure. Modified Hayflick medium (6) were used for the isolation of *M. pneumoniae* from patients. The broth medium was composed of 7.5 parts PPLO broth (Difco), 1.5 parts heat-inactivated horse serum, and 1 part aqueous extract (25%) of baker's yeast, penicillin G (1,000 U/ml), thallium acetate (0.025%), glucose (0.5%), and phenol red (0.002%). The composition of agar medium was the same as that of the broth medium except that glucose and phenol red were omitted and 1.2% agar was added. A throat swab was immersed several times in 0.5 ml of PPLO broth; then, 0.2 ml of the suspension was transferred to the diphasic (agar/broth) medium, and 0.1 ml of the suspension was transferred onto the agar medium. The agar medium was incubated under 5% CO_2 in air with moisture, and the diphasic medium was incubated aerobically at 37°C for 5 to 14 days. When a color change was observed in the diphasic medium, 0.1 ml of the broth was subcultured onto the agar medium. When typical colonies were observed on the agar medium, a single colony was inoculated into the broth medium. After cloning of the colonies, *M. pneumoniae* was identified serologically or by using PCR.

MIC determination. MICs of MLS antibiotics were determined by a broth microdilution method based on the method of the National Committee for Clinical Laboratory Standards. Serial twofold dilutions of MLS antibiotics prepared in PPLO broth containing 10^4 to 10^5 CFU/ml of *M. pneumoniae* were put in 96-well microplates (17). The microplates were sealed with adhesive sheets and incubated at 37°C. The MIC was determined as the lowest concentration of antimicrobial agent at which the color of the control medium was changed. A number of antibiotics were tested. ERY, oleandomycin (OL), josamycin (JM), spiramycin (SPM), midekamycin (MDM), leucomycin (LM), and lincomycin (LCM) were purchased from Wako Pure Chemical Industries, Ltd., Japan; roxithromycin (RXM) and quinupristin-dalfopristin were provided by Aventis Pharm Japan, Ltd.; CLR was provided by Abbott Co., Ltd. (Japan); rokitamycin (RKM) was provided by Asahi Kasei Co. Japan; CLI was provided by Upjohn Co. (Japan); and AZM was provided by Pfizer Japan, Inc.

PCR amplification and sequencing of domains II and V of the 23S rRNA gene and L4 and L22 ribosomal protein genes. The ERY-resistant *M. pneumoniae* strains were screened on the basis of MIC of ERY. A 0.5-ml aliquot of growth culture of *M. pneumoniae* was centrifuged at 17,500 × *g* for 20 min at 4°C. After

TABLE 3. MICs of MLS antibiotics for *M. pneumoniae* isolated from patients and reference strains

Strain no.	23S rRNA mutation[a]	MIC (μg/ml)											
		ERY	OL	RXM	CLR	AZM	JM	MDM	LM	RKM	SPM	LCM	CLI	Q-D[b]
350	A2063G	>256	>256	>256	256	32	8	16	4	0.5	8	>256	>256	1
374	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	>256	256	0.5
375	A2063G	>256	>256	>256	>256	32	16	16	8	0.5	16	>256	256	0.5
376	A2063C	>256	>256	>256	>256	16	64	64	64	4	256	64	32	1
377	C2617G	8	64	8	1	0.031	0.25	0.25	0.25	0.0625	1	16	2	0.25
378	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	256	256	1
379	A2063G	>256	>256	>256	>256	64	8	16	4	0.5	16	256	256	0.5
380	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	0.5
381	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	0.5
382	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	256	256	1
383	A2064G	256	>256	128	32	16	256	>256	>256	32	>256	64	32	0.25
384	A2063G	>256	>256	>256	>256	64	8	16	8	0.5	16	>256	256	1
385	A2063G	>256	>256	>256	>256	64	16	16	16	1	16	>256	256	1
FH		0.0625	0.25	0.0625	0.0156	0.00098	0.0156	0.25	0.0625	0.0625	0.25	16	4	0.0625
M129		0.0156	0.125	0.0156	0.0156	0.00195	0.125	0.0625	0.0625	0.0625	0.125	8	4	0.25
Mac		0.0156	0.25	0.0156	0.0156	0.00098	0.0625	0.0625	0.0625	0.0625	0.0625	4	4	0.25

[a] According to *M. pneumoniae* numbering.
[b] Q-D, quinupristin-dalfopristin.

TABLE 4. Nucleotide substitution by point mutation of genes of ribosomal protein and 23S rRNA for macrolide-resistant *M. pneumoniae* strains and *M. pneumoniae* FH and Mac compared to *M. pneumoniae* M129[a]

Strain no.	Substitution(s) in ribosomal protein						Mutation in 23S rRNA		Type of P1 gene
	Position of L4		Position of L22						
	162	430	62	279	341	508	Domain II	Domain V	
M129	C	A	C	T	C	T	–	–	I
350	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
374	–	–	–	–	–	T→C	–	A2063G	I
375	–	–	–	–	–	T→C	–	A2063G	I
376	C→A	A→G	–	T→C	–	T→C	–	A2063C	II
377	C→A	A→G	–	T→C	–	T→C	–	C2617G	II
378	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
379	C→A	A→G	–	T→C	–	T→C	–	A2063G	II
380	–	–	–	–	–	T→C	–	A2063G	I
381	–	–	–	–	–	T→C	–	A2063G	I
382	–	–	–	–	–	T→C	–	A2063G	I
383	–	–	–	–	–	T→C	–	A2064G	I
384	–	–	–	–	–	T→C	–	A2063G	I
385	–	–	–	–	–	T→C	–	A2063G	I
1020-EMR3	–	–	–	–	–	T→C	–	C2617G	I
1020	–	–	–	–	–	T→C	–	A2064G	I
1253	–	–	C→A	–	C→T	T→C	–	A2064G	I
1552	–	–	–	–	–	T→C	–	A2064C/C2617A	I
1653	–	–	–	–	–	T→C	–	A2064G	I
FH	C→A	A→G	–	T→C	–	T→C	–	–	II
Mac	C→A	A→G	–	T→C	–	T→C	–	–	II

[a] –, No mutation compared to the sequence of *M. pneumoniae* M129.

removal of the supernatant, the sediment was suspended in 20 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer containing 1.0% (vol/vol) Triton X-100 and boiled for 5 min. Specific primers were designed for the detection of the point mutations of domain II of 23S rRNA and of L4 (*rplD*) and L22 (*rplV*) ribosomal proteins (Table 2). Primers for domain V of 23S rRNA were as reported by Lucier et al. (10). To identify the mutation in domain II containing nucleotide A752 interacting with the macrolide 3-cladinose moiety, 23SDIIF-23SDIIR primer pairs were used. For domain V (peptidyltransferase region), MH23SDVF-MH23SDVR primer pairs were used. Amplification of ribosomal protein L4 and L22 fragments was performed with the MNL4F-MNL4R and MNL22F-MNL22R primer pairs, respectively. The composition of the PCR mixture was as follows: 2 μl of template, 30 pmol of forward and reverse primers, and 25 μl of premix *Taq* (TaKaRa Ex *Taq* Version; Takara Bio, Inc.) and water in a final reaction volume of 50 μl. PCR conditions were 2 min at 94°C first, followed by 45 s at 94°C for denaturation, 1 min at 55°C for annealing, and 80 s at 72°C for elongation for 30 cycles, and followed finally by 5 min at 72°C. The

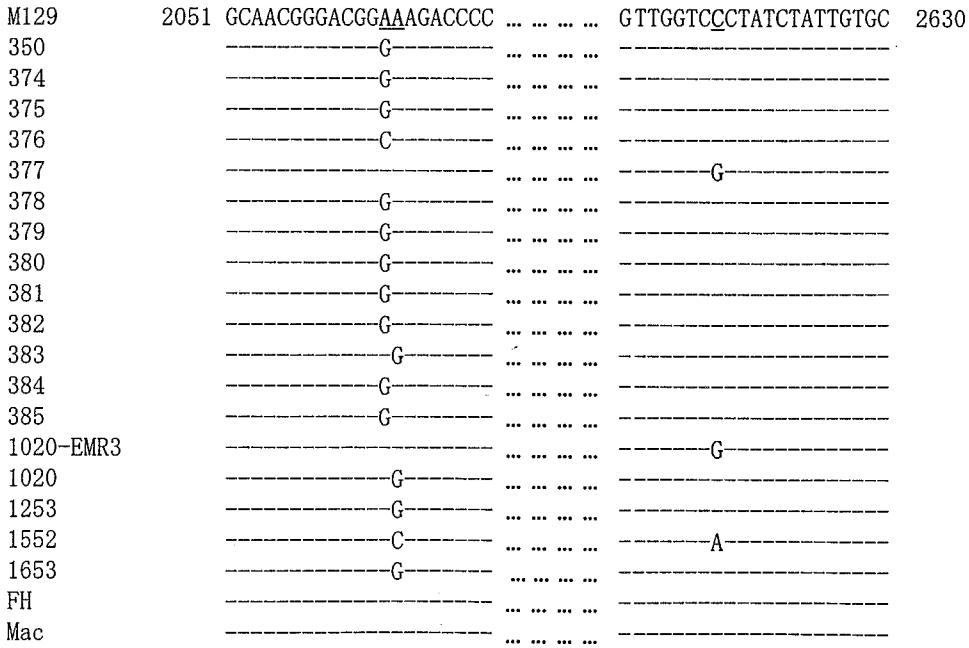

FIG. 1. Multiple alignment of 23S rRNA gene of ERY-resistant *M. pneumoniae* strains and *M. pneumoniae* M129, FH, and Mac. Partial sequences of the peptidyltransferase (domain V) from positions 2051 to 2081 and 2601 to 2630 are presented. The nucleotides are numbered on the basis of *M. pneumoniae*. The nucleotide sequence of *M. pneumoniae* M129 was according to GenBank accession no. X68422. Identical nucleotides are indicated by dashes. The positions of 2063, 2064, and 2617 are underlined.

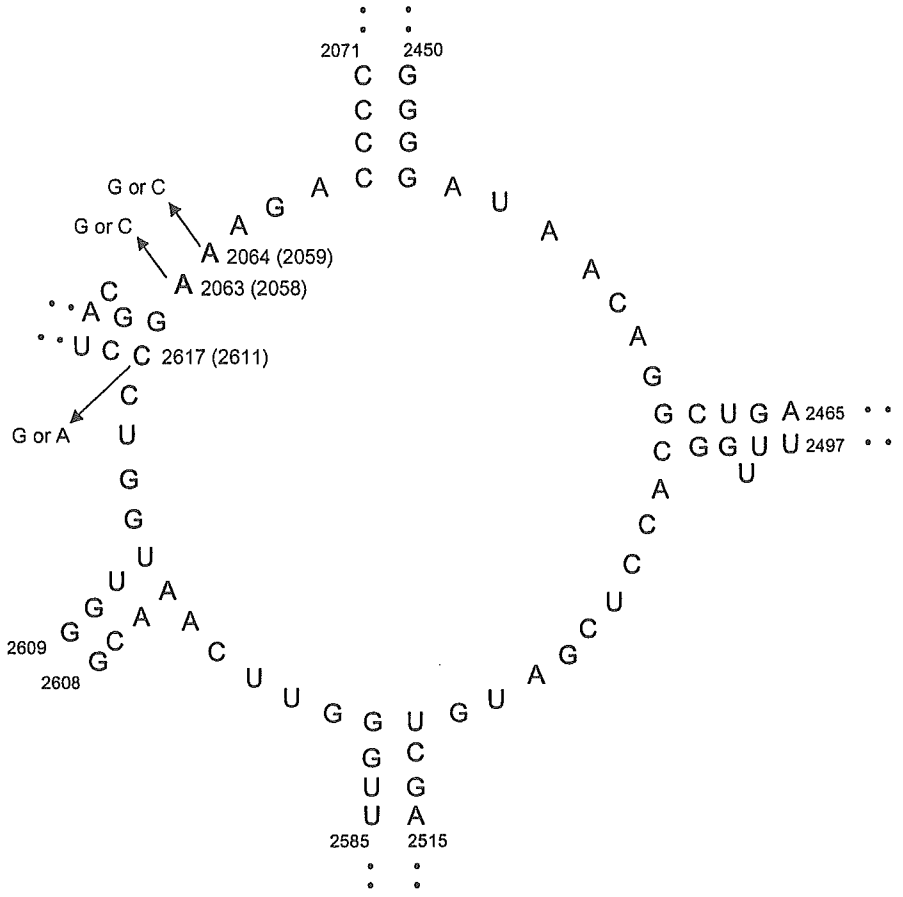

FIG. 2. Secondary structure of the peptidyltransferase loop in domain V of *M. pneumoniae* 23S rRNA. Positions of the newly found mutations (A2063C and C2617G), as well as previously reported in vitro mutations (A2063G, A2064G, and A2064C), in clinical isolates are indicated by using the numbering for *M. pneumoniae* 23S rRNA (accession no. X68422). The numbers in parentheses indicate *E. coli* numbering.

products were purified with a MiniElute PCR purification kit (Qiagen, Hilden, Germany), labeled with a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems), and applied to an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The primers used for sequencing were the same as those used for PCR (Table 2). DNA sequences of PCR products were compared to the sequence of *M. pneumoniae* M129 (accession no. X68422) by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

RFLP analysis of point mutation in domain V of 23S rRNA. To detect the point mutations A2063G, A2063C, A2064G, and A2617G in domain V of 23S rRNA, BbsI, BceAI, BsaI, and BsmFI (New England BioLabs) were used. Second PCR products from domain V for tested *M. pneumoniae* strains were used for digestion with the four restriction enzymes. After the first PCR product (927 bp) was obtained with the MH23SDVF-MH23SDVR primer pair, a second PCR product (210 bp) was obtained with the MN23SF1937-MN23SR2128 primer pair to detect the point mutation at 2063 or 2064 in domain V of 23S rRNA. For the detection of point mutation at 2617 in domain V, the primer set of MN23SF2577 and MN23SF2664 was used, and a 108-bp PCR product was obtained. A portion of the second PCR product was digested with BbsI (5 U for 1 μl of PCR product) for the A2063G mutation, BceAI (1 U for 1 μl of PCR product) was used for the A2063C mutation, BsaI (10 U for 1 μl of PCR product) was used for the A2064G mutation, and BsmFI (2 U for 1 μl of PCR product) was used for the C2617G mutation. Digested products were electrophoresed on a 10 to 15% gradient polyacrylamide gel (Nikkyo Technos Co., Ltd.) or on a 4% Nusieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, Maine).

RESULTS

Antimicrobial susceptibility. In all, 13 (17%) of the 76 clinical isolates obtained in Japan during the period from 2000 to 2003 showed various degrees of elevation of MICs against macrolides, including the ERY MIC. The in vitro activities of the MLS antibiotics against ERY-resistant clinical isolates and reference strains of *M. pneumoniae* are summarized in Table 3. *M. pneumoniae* reference strains, including M129, showed low ERY, OL, RXM, CLR, AZM, JM, MDM, LM, RKM, and SPM (0.0156 to 0.25 μg/ml) MICs. Of the ERY-resistant strains, strain 377 (C2617G) showed low resistance to macrolide antibiotics except for OL. The 15-membered macrolide AZM and most of the 16-membered macrolides were more effective than the 14-membered macrolides for strain 377. Although ERY-resistant clinical strains, except for strain 377, tended to show resistance to all of the macrolides, some of them showed different responses to RKM. That is, for strains with an A-to-G mutation at position 2063 the RKM MICs were not so high (<1 μg/ml). LCM and CLI, lincosamide antibiotics, and streptogramin antibiotics showed no marked activity toward the reference strains or some of the clinical isolates.

Sequencing analysis of ribosomal protein and 23S rRNA genes. PCR amplification and sequence analysis of ribosomal proteins and 23S rRNA were performed for all *M. pneumoniae* strains used in the present study. The results are summarized in Table 4. In domain II of the 23S rRNA containing position 752, there was no difference in sequence from that of *M.*

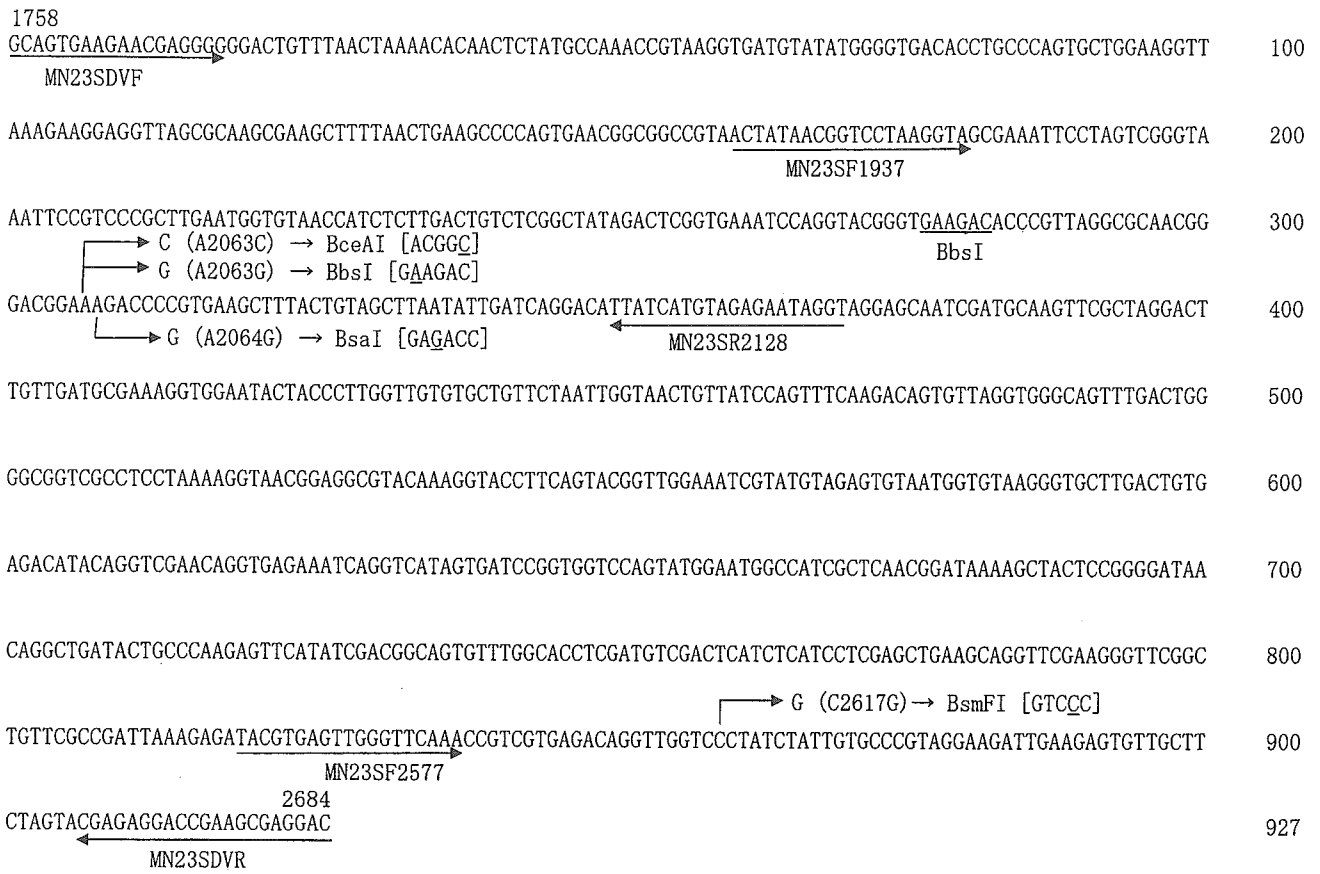

FIG. 3. Nucleotide sequence of the 927-bp amplicon from positions 1758 to 2684 of the 23S rRNA gene from *M. pneumoniae* M129. A long arrow indicates a primer sequence with direction. A short arrow indicates a site of mutation with a substituted base, i.e., A2063G, A2063C, A2064G, or C2617A. A newly constructed restriction site and the responsible base change with underline is shown in parentheses with the corresponding restriction enzyme.

pneumoniae M129. Figure 1 shows the results of the nucleotide sequence analysis of domain V, called the peptidyltransferase region, in the 23S rRNA of the *M. pneumoniae* strains. Five ERY-resistant strains (1020-EMR3, 1020, 1253, 1552, and 1653) were induced with ERY in vitro, as previously reported (17). Figure 2 shows the position of a point mutation on the peptidyltransferase loop in domain V of *M. pneumoniae* 23S rRNA. Of 13 ERY-resistant clinical isolates, 10 (77%) showed A2063G transition, and the remaining 3 showed one A2064G transition, one A2063C transversion, and one A2617G transversion. Of the ERY-resistant strains obtained in vitro, strain 1020-EMR3 had C2617G and strain 1552 had two point mutations: A2064C and C2617A. Compared to the sequence of the M129 strain, different nucleotides were found in some strains (350, 376, 377, 378, 379, FH, and Mac) at positions 162 and 430 of L4 and 279 of L22 ribosomal protein genes. These differences are related to two different types of *M. pneumoniae* strains (19). Mutation T508C of the L22 ribosomal protein gene was observed in all strains used in the present study except for M129. Thus, these nucleotide differences are not involved in the ERY resistance of *M. pneumoniae*. Although C62A and C341T mutations were found in strain 1253, it is uncertain whether these mutations are involved in ERY resistance because of the A2064G mutation, which imparts high ERY resistance.

RFLP analysis of ERY-resistant *M. pneumoniae* strains. To detect a point mutation at position 2063 or 2064 of the 23S rRNA gene, a second PCR product (210 bp) was digested from the first PCR product (927 bp) with suitable restriction enzymes. Digestion with BsaI generated two fragments of 124 and 86 bp for ERY-susceptible strain M129, whereas three fragments of 124, 57, and 29 bp were obtained in the case of the A2063G mutation (lanes 2 and 3 in Fig. 4A). Two fragments of 158 and 52 bp were generated with BceAI in the case of the A2063G mutation (lane 5 in Fig. 4A), and two fragments were generated with BsaI in the case of the A2064G mutation (lane 7 in Fig. 4A). Strain M129 has no cut site for the second PCR product with BceAI and BsaI (lanes 4 and 6 in Fig. 4A). To detect a point mutation at position 2617, the PCR primer pair MN23SF2577 and MN23SDVR was used, generating a 108-bp product (Fig. 3). Although there was no restriction enzyme to digest C2617A or C2617G mutation, the M129 strain had a restriction site with BsmFI and generated two fragments of 81 and 27 bp (Fig. 4B).

DISCUSSION

In general, macrolides such as ERY, CLR, and AZM are used as the first-choice therapeutic agent for treating *M. pneumoniae* infections in children, as well as in adults. We isolated

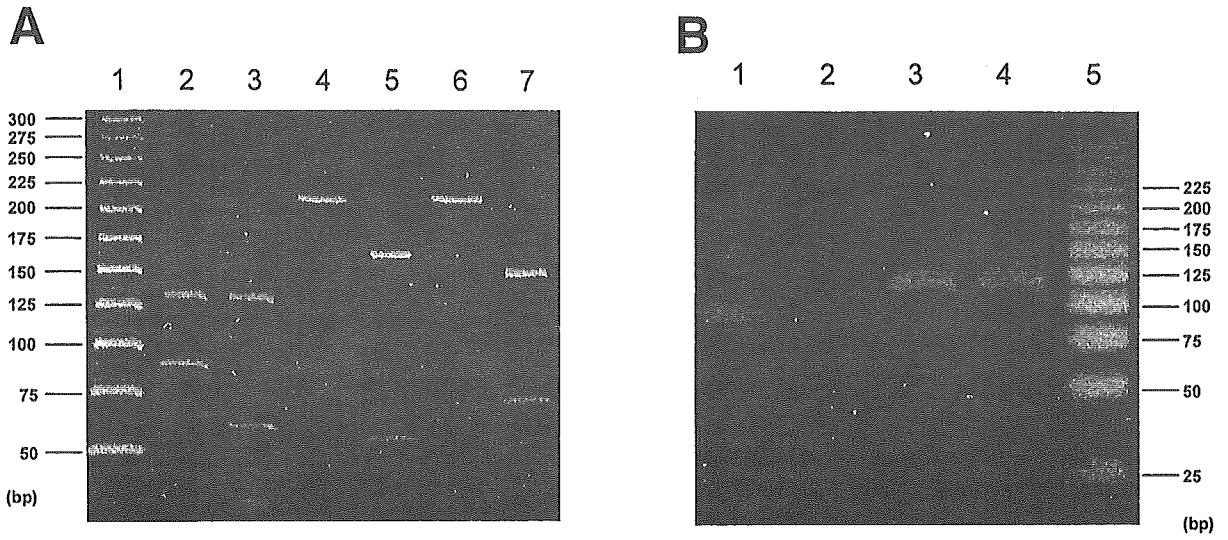

FIG. 4. Restriction analysis of 210-bp (A) and 108-bp (B) amplicons from the peptidyltransferase region (domain V) in 23S rRNA of *M. pneumoniae*. (A) Restriction profile for detection of the A2063G, A2063C, and A2064G mutations. Lanes: 1, DNA size marker (25-bp DNA step ladder; Promega); 2, 4, and 6, *M. pneumoniae* M129 (susceptible strain) treated with BbsI (lane 2, 124-, and 86-bp products) and BceAI and BsaI (lanes 4 and 6, respectively; uncut 210-bp product); 3, strain 375 (A2063G) treated with BbsI (124-, 57-, and 52-bp products); 5, strain 376 (A2063C) treated with BceAI (158- and 52-bp products); 7, strain 1020 (A2064G) treated with BsaI (141- and 69-bp products). (B) Restriction profile for detection of C2617 mutation with BsmFI digestion. Although *M. pneumoniae* M129 and strain 375 (A2063G) produced two fragments of 81 and 27 bp (lanes 1 and 2), the 108-bp fragment remained uncut in strains 377 and 1020-EMR3 (C2617G) as a result of loss of the restriction site for BsmF1 (lanes 3 and 4). Lane 5, DNA size marker (25-bp DNA step ladder; Promega).

76 *M. pneumoniae* strains from three geographically distant regions in Japan (Hokkaido in the northern island, Kanagawa in the central region, and Kochi in south) and found that 13 strains (17%) were ERY resistant. Although resistance to ERY was observed many years ago in a few *M. pneumoniae* strains (16, 20), when we investigated the ERY MICs for 296 *M. pneumoniae* strains isolated in Japan from 1983 to 1998, no ERY-resistant strain was found among them (data not shown). Thus, we concluded that ERY-resistant *M. pneumoniae* had appeared in 2000 and spread rapidly in Japan. We applied our established RFLP analysis to ca. 1,000 sputum samples taken from patients with respiratory infections from 2000 to 2002 and found that 23 (24%) of 94 PCR-positive samples for *M. pneumoniae* DNA had the ERY resistance-inducing point mutation A2063G (unpublished data). Whether or not the prevalence of ERY-resistant *M. pneumoniae* and the predominance of A2063G among the isolates are peculiar to Japan needs to be clarified by future studies outside Japan.

The mechanisms of resistance to MLS antibiotics in various microorganisms have been reviewed and include modification of the target site, active efflux, or inactivation (13, 24–26). The MLS antibiotics inhibit protein synthesis by binding to domains II and V of 23S rRNA (3, 26). In particular, it has been clearly shown that ribosomal mutations in domains II and V of 23S rRNA and mutations in ribosomal protein L4 (*rplD*) and L22 (*rplV*) are related to resistance to MLS antibiotics (2, 4). In L4 and L22 ribosomal proteins, no mutation that clearly contributed to resistance to macrolide antibiotics was found, although one strain (strain 1253) exhibited mutations of the L22 protein, such as C62A and C341T, in vitro. We found several point mutations in domain V of 23S rRNA in ERY-resistant *M. pneumoniae* but none in domain II of 23S rRNA. Among them, the point mutations at position 2063 or 2064 in domain V have been reported in several pathogens such as *E. coli*, *H. pylori*, *Mycobacterium* spp., and *S. pneumoniae* (24) and generated strong resistance to macrolide antibiotics. Transversions of C to G and C to A at position 2617 of domain V were observed in a clinical isolate (strain 377) and ERY-induced strains (1020-EMR3 and 1552), respectively. On the other hand, it has been reported that C-to-U transition at position 2611 (corresponding to 2617 in *M. pneumoniae* numbering) in clinical pathogens such as *Neisseria gonorrhoeae* (15), *Streptococcus pyogenes* (11), *Mycoplasma hominis* (18), *Chlamydia trachomatis* (12), and *E. coli* (23) was associated with macrolide resistance. *M. pneumoniae* strain 1552, derived by incubation with ERY in vitro, showed A2064C transversion and C2617A transversion. The mutation at position 2617 produced less resistance to macrolide antibiotics than did the mutation at position 2063 or 2064 of domain V. Based on our results, it is considered that transition is the predominant type of mutation in *M. pneumoniae*. This may be due to the structural difference between purine and pyrimidine. These results support the observation in *E. coli* that the apparent dissociation constant (K_d) for ERY of C2611U (corresponding to 2617 in *M. pneumoniae*) [$K_d = (4.4 \pm 0.9) \times 10^{-7}$] is ca. 480 times higher than that of the A2058G (2063 in *M. pneumoniae*) *E. coli* strain [$K_d = (1.9 \pm 0.3) \times 10^{-4}$] (3). As mentioned above, macrolide resistance of *M. pneumoniae* has been explained thus far in terms of mutation of 23S rRNA. However, *M. hominis* was associated with an absence of intracellular accumulation and ribosomal binding of macrolide antibiotics (18). These results suggest that several different mechanisms of macrolide resistance exist in *Mycoplasma* species.

Table 1 summarizes information about the patients from whom ERY-resistant *M. pneumoniae* strains were isolated. Although these patients were actually infected with ERY-resis-

tant *M. pneumoniae*, macrolides were apparently effective after their first administration in six (ERY in cases 383 and 384, CLR in case 350, and AZM in cases 377, 378, and 381) of the ten patients for whom the clinical course was known. One possible explanation may be the anti-inflammatory effects of macrolides, which inhibit the production of cytokines such as proinflammatory tumor necrosis factor alpha, interleukin-1β (IL-1β), IL-6, IL-8, and so on rather than the antimicrobial effect (1, 7, 8, 21). Much more information is available about the immunopathological mechanisms of *M. pneumoniae* pneumonia, particularly with regard to a wide variety of cytokines. Among them, Th1-type cytokines (22) and IL-8 (14) might play significant roles in the pathomechanism. In this context, recent investigations have revealed that macrolides modulate the actions of these cytokines (5, 9). It is therefore a reasonable proposition that macrolides, particularly 14- and 15-membered macrolides, exert their clinical efficacy in the treatment of *M. pneumoniae* pneumonia through immunomodulation. Our results obtained for patients with ERY-resistant *M. pneumoniae* infection strongly suggest that the beneficial effects of macrolides in the treatment of *M. pneumoniae* pneumonia are not solely due to direct antimicrobial activity and support the idea that immunomodulatory effects of macrolides play an important role in recovery from the illness.

In conclusion, we found 13 strains of macrolide-resistant *M. pneumoniae* among 76 clinical isolates obtained during the period from 2000 to 2003, despite the fact that no resistant strain was found among 296 isolates from 1983 to 1998. The predominant mutation was A2063G in domain V of 23S rRNA (10 of 13 resistant strains), and mutations involving either A2063 or A2064 resulted in high MICs to macrolide antibiotics. On the other hand, mutations involving C2617 in domain V of 23S rRNA generated less resistance to ERY than mutations involving A2063 or A2064. Our results indicate that macrolide-resistant *M. pneumoniae* is spreading in Japan, and it will be necessary to reconsider the effectiveness of macrolides in the treatment of patients with *M. pneumoniae* pneumonia.

ACKNOWLEDGMENTS

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Current status of macrolide-resistant *Mycoplasma pneumoniae* in Japan.

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Key words : 23S ribosomal RNA, immunomodulatory action, anti-inflammatory effects

Abstract.

Four cases with *Mycoplasma pneumoniae* pneumonia were presented from which macrolide-resistant *M. pneumoniae* strains were isolated. Three kinds of a point mutation were identified in 23S rRNA of the isolates. Despite of having those mutations in their isolates, the patients were successfully treated by a macrolide therapy. As many as 17% of circulating strains of *M. pneumoniae* are macrolide-resistant currently in Japan. Nevertheless, apparently no serious problems have been reported to date that can be ascribed to those resistant strains. The efficacy of macrolides in improving the clinical picture of *M. pneumoniae* pneumonia, specifically concerning their immunomodulatory action with anti-inflammatory effects, is discussed.

Introduction.

Only a few studies on macrolide-resistant *M. pneumoniae* have been reported to date, and the clinical manifestations of infection with resistant strains of *M. pneumoniae* have not been sufficiently described. In this point, the author obtained a macrolide-resistant strain of *M. pneumoniae* for the first time in 2000 in Sapporo, subsequently in Ikeda in 2002, and noted that the clinical course of the patients did not necessarily indicate macrolide-resistance.

Case reports.

Case 1 was a 9-year-old girl having pneumonia in the left middle lung field diagnosed by her primary physician. Despite 7 days of treatment with intravenous clindamycin, the symptoms of fever and coughing continued and she developed right upper lobe pneumonia as well. The treatment was changed to an oral clarithromycin therapy and her sustained fever regressed sharply within 2 days and the abnormalities in chest radiograph improved in a week. Case 2 was a 12-year-old girl who was diagnosed as having left upper lobe pneumonia at the Ikeda Municipal Hospital. Her initial treatment with clarithromycin which had not been effective was replaced by azithromycin on the 3rd day of illness, and her fever subsided gradually taking over 3 days during the azithromycin treatment. Case 3 was a 2-year-old girl who developed fever and coughing and was initially treated by cefditoren-pivoxil, an oral cephem, for 4 days without improvement. The treatment was changed to azithromycin therapy on the 5th day of illness when pneumonia was found in the left middle lung field. Her fever regressed next day. Case 4 was a 7-year-old girl who developed fever and coughing and came to the Ikeda Municipal Hospital. Her fever regressed within 2 days following the commencement of azithromycin therapy.

Methods.

For isolation of *M. pneumoniae,* throat swab samples were obtained at the first visit to hospitals and inoculated into PPLO liquid medium. Minimal inhibitory concentrations (MICs) were determined at the Kanagawa Prefectural Public Health Laboratories by a micro dilution method based on NCCLS. An MIC was recorded as the lowest concentration of antibiotic that suppressed the growth of *M. pneumoniae* when the color of the growth control medium changed. 23S rRNA gene sequences were determined at the National Institute of Infectious Diseases in Musashi-murayama by direct sequencing of PCR products.

Results.

Results of susceptibility testing for the isolates obtained from the 4 patients (designated as isolates 1-4) along with other susceptible isolates are shown in Table 1.

Unexpectedly in case 1, the isolate was resistant to clarithromycin which was clinically effective, and similarly in cases 2 and 3, the isolates were resistant to azithromycin *in vitro.* Isolate 4 showed various degrees of increases in MICs for macrolides. Direct sequencing of the domain V of 23S rRNA gene detected a point mutation of A2063G transition for isolates

Table 1. MICs of macrolides and other antibiotics for *M. pneumoniae* clinical isolates.

Antibiotics	Isolate 1 (A2063G)	Isolate 2 (A2063C)	Isolate 3 (A2063G)	Isolate 4 (C2617G)	Others (n=9)
Erythromycin	>12.5	>12.5	>12.5	3.125	0.006-0.012
Clarithromycin	>12.5	>12.5	>12.5	0.78	0.00048-0.003
Oleandomycin	>12.5	>12.5	>12.5	>12.5	0.049-0.195
Roxithromycin	>12.5	>12.5	>12.5	12.5	0.00152-0.003
Azithromycin	>12.5	>12.5	>12.5	0.012	0.000012
Josamycin	12.5	>12.5	12.5	0.049	0.006-0.049
Spiramycin	12.5	>12.5	12.5	0.78	0.012-0.195
Midecamycin	>12.5	>12.5	12.5	0.195	0.012-0.049
Kitasamycin	4	>12.5	4	0.195	0.006-0.012
Clindamycin	>12.5	>12.5	>12.5	2	3.125->12.5
Tetracycline	0.39	0.39	0.39	0.78	0.195-0.78
Minocycline	Not tested	0.098	0.098	0.39	0.098-0.39

1 and 3, A2063C transversion for isolate 2, and C2617G transversion for isolate 4. A2063 is corresponding to A2058 in *Escherichia coli,* and C2617 corresponding to C2611.

Including the 4 strains presented above, a total of 13 strains of macrolide-resistant *M. pneumoniae* have been obtained (Table 2). A strain with A2064G transition was recently found. As many as 17% of circulating strains of *M. pneumoniae* were macrolide-resistant. Places of isolation are geographically distant from each other, therefore, this number of occurrence must represent the ubiquitous status in Japan. From the clinical point of view, not a single doctor who saw the patients in unrelated hospitals felt that macrolides were effective in 8 out of the 11 cases with resistant strain infection for which clinical records were available.

Discussion.

Two points must be emphasized from this study. Firstly, the frequency of resistant strains may actually have been increasing in recent years, because, among 296 isolates obtained in Japan during 1983-98, we could not find any resistant strain. Secondly, our routine drug susceptibility testing for *M. pneumoniae* clinical isolates might contribute to detect indwelling mutants such as isolates 3 and 4, for which, the clinical course did not show any drug resistance. This may have a significant implication as regards to the pathomechanism of *M. pneumoniae* pneumonia.

In this context, a concept of immunopathogenesis of *M. pneumoniae* pneumonia must be important. A glycolipid membrane of *M. pneumoniae* is a potent inducer of cytokines and much information is available about the immunopathological mechanisms of *M. pneumoniae* pneumonia, particularly as regards to a wide variety of cytokines. Among them, the author and coworker, TANAKA Hiroshi of the third Department of Internal Medicine, Sapporo Medical University, have presented the data that IL-18 and IL-8 must play significant roles in the immunopathological mechanism. For this point, recent investigations have revealed that 14-and 15-membered-ring macrolides can modulate the actions of these cytokines. It is, therefore, a reasonable assumption that macrolides exert their therapeutic effects on the treatment of *M. pneumoniae* pneumonia not only by their anti-microbial activity but also through their immunomodulatory action with anti-inflammatory effects.

Following the infection, *M. pneumoniae* slowly increases in number, taking 2 or 3 weeks of incubation. When the amount of bacteria reaches a certain level, a host recognizes the infection and elicits immune responses, initially by cytokines which may be evoked within hours, and later by specific antibody production which may take place within days. With the initiation

Table 2. Macrolide resistant *M. pneumoniae* isolated from patients and their information.

Mutation	Patient		Antimicrobial agents		Hospital/ Institute
	Age	Symptoms/Disease	1 st choice/Effect	2 nd choice/Effect	
A2063G	9	Pneumonia	Clindamycin/−	Clarithromycin/+	Sapporo
	3	Pneumonia	Unknown		Kochi
	4	Pneumonia	Unknown		Kochi
	2	Pneumonia	Cefditoren Pivoxil/−	Azithromycin/+	Sapporo
	9	Pneumonia	Clarithromycin/−	Azithromycin/−	Chigasaki
	11	Pneumonia	Clarithromycin/−	Minocycline/+	Chigasaki
	11	Pneumonia	Azithromycin/+		Chigasaki
	7	Pneumonia	Rokitamycin/−	Azithromycin/−	Chigasaki
	7	Pneumonia	Cefdinir, Fosmicin/−	Erythromycin/+	Chigasaki
	?	Pneumonia, Pleuritis	Clarithromycin/+		Kanagawa
A2063C	12	Pneumonia	Clarithromycin/−	Azithromycin/+	Ikeda
A2064G	5	Bronchitis	Cefaclor/−	Erythromycin/+	Chigasaki
C2617G	7	Fever and Cough	Azithromycin/+		Ikeda

Effect; +; Clinically improved, −; Clinically no effect

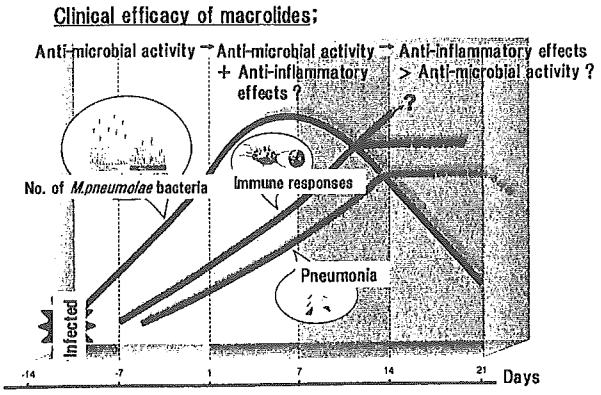

Figure. Hypothetical presentation of clinical efficacy of macrolides in the treatment of *M. pneumoniae* pneumonia.

of these host immune responses, propagation of *M. pneumoniae* becomes to be suppressed and at the same time, on the other hand, the pathological feature of pneumonia appears. Later in the course, the host immune responses achieve a plateau, pneumonia begins to resolve, and it disappears usually with a 2 or 3 weeks of self-limited natural course. Our results strongly suggest that the beneficial effects of macrolides for the treatment of *M. pneumoniae* pneumonia are not solely dependent on their direct antimicrobial activity, and the author believes that the immunomodulatory action must play an important role. That is, in the early phase of infection, certainly, the anti-microbial activity is critical to suppress the propagation of *M. pneumoniae.* In the middle phase of infection, the immunomodulatory action becomes to be necessary. In the later phase of infection, the anti-inflammatory effects, even more than the anti-microbial activity, may become important for the clinical efficacy of macrolides in terms of improving the clinical picture of pneumonia. The efficacy of macrolides unexpectedly observed in the course of macrolide-resistant *M. pneumoniae* pneumonia can be explained by this scenario.

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本誌に掲載された統計資料は，1)「感染症の予防及び感染症の患者に対する医療に関する法律」に基づく感染症発生動向調査によって報告された，患者発生および病原体検出に関するデータ，2) 感染症に関する前記以外のデータに由来する。データは次の諸機関の協力により提供された：保健所，地方衛生研究所，厚生労働省食品安全部，検疫所，感染性腸炎研究会。

＜特集＞ デング熱・デング出血熱輸入症例 1999.4～2003.12

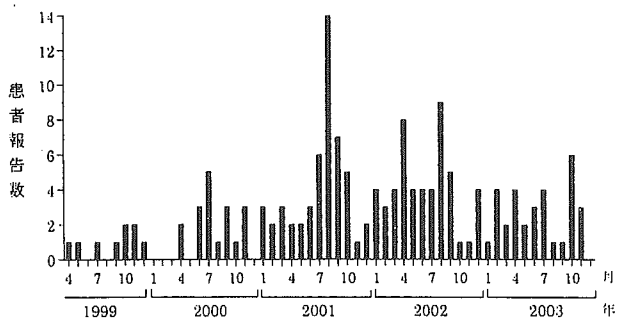
デングウイルスはネッタイシマカやヒトスジシマカの刺咬によりヒトに感染する（本号9ページ参照）。デングウイルス感染により，デング熱とデング出血熱/ショック症候群という2つの異なる病態を示す。デング熱は，発熱・発疹・痛み（関節痛）が3主徴であるが致死率は低い。これに対して，1953年にフィリピンで初めて確認されたデング出血熱は，発熱・出血傾向・循環障害を示し，適切な治療を行わないとショック死する危険性が高い。わが国ではデング熱は1942～1945年にかけて西日本（長崎，佐世保，広島，呉，神戸，大阪）の諸都市で流行したことが報告されている。ウイルスは東南アジアから軍用船で帰国したデング熱患者によって国内に持ち込まれ，国内に生息するヒトスジシマカにより流行が引き起こされたと考えられている。現在，日本国内にはデングウイルスは常在せず，国内での感染はない。しかし，デングウイルスが常在する熱帯・亜熱帯地域の渡航先でデングウイルスに感染し，帰国後発症する輸入例が毎年相当数存在する(本月報Vol.21, No.6参照)。また，流行地域からの入国者がわが国で発症する例もみられる。

表1．デング熱患者報告数，1999～2003年

診断年	男性	女性	合計
1999	7	2（1）	9（1）
2000	11	7	18
2001	31（2）	19	50（2）
2002	32（2）	19（1）	51（3）
2003	20（2）	11	31（2）
計	101（6）	58（2）	159（8）

1999年は4～12月 （）内はデング出血熱例数再掲
（感染症発生動向調査：2004年1月7日現在報告数）

図1．デング熱・デング出血熱患者発生状況，1999年4月～2003年12月



（感染症発生動向調査：2004年1月7日現在報告数）

感染症発生動向調査：1999年4月に施行された感染症法では，デング熱は全数把握の4類感染症に指定され，2003年11月の法改正後には同じく全数把握の新4類に分類されている。感染症法施行後に届けられたデング熱患者は159例で，全例輸入例であった。1999年（4～12月）9例，2000年18例と少なかったが，2001年は50例，2002年は51例と増加した。2003年は31例であった（表1）。2001～2002年に増加したのは，世界的な流行を反映していると思われるが，全数届け出疾患となったデング熱に対する医師の認識が向上し，デング熱と診断される症例が増加したことも影響していると考えられる。

月別患者発生状況は，渡航先の流行時期および日本からの海外旅行者数の変動，の二つの要因の影響を受けると考えられる。患者が増加した2001～2002年は，夏季または春季に多い傾向が認められたが，2003年は重症急性呼吸器症候群（SARS）発生地域への渡航制限が海外旅行全体に影響を及ぼしたためか，そのよう

表2．デング熱患者の渡航先の内訳，1999～2003年

	1999	2000	2001	2002	2003	計
アジア						
タイ	2	3	23	18	6	52
インドネシア	1	3	10	13	7	34
フィリピン	1	3	8	5	1	18
インド	3	5	-	-	5	13
カンボジア	-	-	4	2	-	6
ミャンマー	-	-	1	3	2	6
マレーシア	-	1	1	2	1	5
バングラデシュ	2	1	-	1	-	4
ラオス	-	1	2	-	-	3
スリランカ	-	-	1	1	1	3
ベトナム	-	-	1	1	-	2
東チモール	-	-	-	1	-	1
ネパール	1	-	-	-	-	1
オセアニア・南太平洋						
ニューカレドニア	-	-	-	-	3	3
クック諸島	-	-	-	2	-	2
フィジー	-	-	-	-	2	2
仏領ポリネシア	-	-	2	-	-	2
パプアニューギニア	-	-	-	-	1	1
ミクロネシア	-	-	1	-	-	1
中南米						
ブラジル	-	-	1	2	1	4
コスタリカ	-	-	1	-	-	1
ボリビア	-	-	-	-	1	1
ドミニカ共和国	-	1	-	-	-	1
エクアドル	-	-	-	-	1	1
パラグアイ	1	-	-	-	-	1
例数	9	18	50	51	31	159

*2か国以上を含む。 1999年は4～12月。
（感染症発生動向調査：2004年1月7日現在報告数）

（2ページにつづく）

していたため，感染症および食中毒の両面から調査が進められた。なお，他の宿泊グループのなかには発症者はいなかった。

検査材料・方法：入院患者の咽頭ぬぐい液12検体，宿泊施設の調理従事者の咽頭ぬぐい液4検体，保存検食7検体，調理場のふきとり10検体について，血液寒天培地を用いて，直接およびSEB培地（ニッスイ）による選択増菌した後，塗抹し37℃24時間培養した。血液寒天培地上でβ溶血環を示したコロニーについて，アピストレップ20（bio Mérieux）で生化学的性状を確認し同定を行い，ストレプトコッカス群別キット「ユニブルー」（オクソイド社）で群を決定した。T型は，T型別用免疫血清（デンカ生研）を使用して型別した。

結果：当センターにおける検査で，患者の咽頭ぬぐい液12検体中10検体から *Streptococcus pyogenes* T28型（発熱性毒素B＋C産生）が検出された。宿泊施設の調理従事者4名の咽頭ぬぐい液，保存検食7検体，調理場のふきとり10検体から *S. pyogenes* は検出されなかった。

当該宿泊施設の調理従事者の咽頭および保存検食から菌は検出されず，他の宿泊グループからも発症報告はなかったことから，宿泊施設での食中毒は否定されたが，発症者数が各組ほぼ同程度であったこと，9～10日に集中した一峰性の患者発生パターンであったことから，本事例は単一曝露による集団感染の可能性が高いと考えられた。

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マクロライド耐性 *Mycoplasma pneumoniae* 増加の兆し

マイコプラズマは自己増殖可能な最小の微生物で生物学的には細菌に分類されるが，他の細菌と異なり細胞壁を欠くため多形態性を示し，ペニシリン，セフェム等の細胞壁合成阻害剤には感受性を示さない。マイコプラズマ肺炎は臨床的にクラミジア肺炎と類似しているため，治療においては両者に有効なテトラサイクリン系やマクロライド系の抗菌薬が一般に使用されているが，小児，特に新生児や乳児に対しては，下痢，大泉門の膨隆，骨の発育障害，黄歯などの副作用の観点からテトラサイクリン系薬剤より，エリスロマイシン，クラリスロマイシン，などのマクロライド系薬剤やクリンダマイシン（リンコマイシン系薬剤）を投与するのが一般的とされている。

これまで国内で分離された *Mycoplasma pneumoniae* に関するマクロライド薬剤に対する感受性成績が日本マイコプラズマ学会や日本感染症学会に報告されてきたが，少なくとも5年前まではマクロライド耐性 *M. pneumoniae* の報告はなかった。しかし，この3～4年前よりマクロライド耐性 *M. pneumoniae* の分離が目立つようになってきた。

表1は北海道，高知県，神奈川県で分離されたマクロライド耐性 *M. pneumoniae* の比率と耐性獲得に関与する23S rRNA遺伝子の変異パターンを示す。これらのマクロライド耐性 *M. pneumoniae* は，14員環（エリスロマイシン，オレアンドマイシン，ロキシスロマイシン，クラリスロマイシン），15員環（アジスロマイシン），16員環（キタサマイシン，ジョサマイシン，ミデカマイシン）マクロライドのいずれにも耐性を示すので，治療においては注意を要する。

現在のところ，マクロライド耐性 *M. pneumoniae* 感染症がとりわけ重症化しやすいという傾向は必ずしも認められておらず，通常の感受性菌による感染と臨床的に鑑別することは極めて難しい。したがってその治療はどうしても主治医の判断による経験的な治療にならざるを得ない。小児においては，14員環または15員環マクロライド剤の使用が主流である。ただ，マクロライド耐性 *M. pneumoniae* 感染患者においてもクラリスロマイシンあるいはアジスロマイシンを投与することによって症状が回復したケースはあった。一方，成人においては基本的には投薬のしばりが無いので，ミノサイクリン，シプロフロキサシン，ガチフロキサシン，スパルフロキサシン，レボフロキサシンなどから状況に応じて適切な薬剤を選択すればよいと考えら

表1．患者分離マクロライド耐性 *M. pneumoniae* 株の特徴

分離	分離年月	耐性株／分離株	耐性菌分離比率（%）	23S rRNA遺伝子変異パターン*
北海道	2000/10～2002/11	4/26	15.4	A2063G, A2063G, C2617G, A2063C
高知県	2001/9-10	2/6	33.3	A2063G, A2063G
神奈川県	2003/8-11	7/41	17.1	A2063G, A2063G A2063G, A2063G A2063G, A2063G A2064G

＊．*M. pneumoniae* 23S rRNA遺伝子番号を示し、A2063Gは2063番目のAがGに変異を起こしていることを示す。

れる。

In vitro 試験で確かめた結果，23S rRNA 遺伝子の2063または2064番目のA（アデニン）がG（グアニン）またはC（シトシン）に変るとマクロライド系に高い薬剤耐性を示す。また，2617のCがGに置換した株も1株分離されたが，*in vitro* 試験においても本置換は低～中程度の耐性獲得に関与していることが明らかになった。

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23価肺炎球菌ワクチンの感染防止効果および医療経済効果について

肺炎球菌は呼吸器感染症の主要な起炎菌であり，肺炎症例の約30％は、この菌によって引き起こされると考えられている[1]。肺炎球菌感染症に対するワクチンとしては、この菌が持つ莢膜を利用した23価多糖体ワクチンおよび，7価コンジュゲートワクチンがあるが，わが国で接種可能なワクチンは前者のみである。23価肺炎球菌ワクチン接種により，多糖体に対する抗体価の上昇は見られるが[2]，実際に感染防止効果があるかどうかという点に関しては，長い間，結論が得られていなかった。このワクチンの添付文書の効能・効果欄には，「肺炎球菌による感染症の予防」と記されている。Jackson らは，47,365人の65歳以上の高齢者に対して，3年にわたる後ろ向きコホート研究を行い，2003年にその結果を公表した[3]。その結論は，23価肺炎球菌ワクチンは，肺炎球菌による菌血症（菌が血液から分離される状態）がおこる割合を1,000人・年あたり，0.68（ワクチンを接種しなかった場合）から0.38（ワクチンを接種した場合）に44％減少させることができるが，肺炎に対する防止効果は見られないというものであった。1998年には，23価肺炎球菌ワクチンには肺炎防止効果が見られないという二重盲研試験の結果も発表されている[4]。

また，医療経済効果に関する検討では，米国において，23価肺炎球菌ワクチンの接種料金＄12という前提で，1接種あたり，＄6.68から＄10.91の経済効果があるとされている[5]。なお，日本における23価肺炎球菌ワクチンの薬価は￥5,053となっているので，米国と同様に論じることはできない。

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国立感染症研究所・細菌第一部
和田昭仁　倉　文明　前川純子
池辺忠義　常　彬　渡辺治雄

＜外国情報＞

クイーンズランド州北部でのデング熱，2002年――オーストラリア

クイーンズランド州の北部では，国内感染によるデング熱患者発生の頻度が顕著に増加している。本報告では，2002年のこの地域におけるデング熱の発生状況について記載した。

Kuranda におけるデングウイルス2型患者の発生： 2002年3月上旬，Kuranda 在住の成人男性が，また中旬には同在住の成人女性が，いずれも EIA によりデング陽性との届け出があった。両者とも Kuranda から外部への旅行歴はなかったが，女性患者はデング熱様症状を有しており，彼女が働いている地元のホテルには同様の症状を有している従業員が他にも数人いた。調査の結果，合計で21例がデング熱と確定し，そのうちの3例からデングウイルス2型が同定された。21例中18例はホテルの従業員または宿泊客（8例），ホテル付近に勤務または在住（10例）のいずれかであった。

デングウイルスE遺伝子の分子生物学的解析では，タイから輸入されたデングウイルス2型に類似していた。

Townsville におけるデングウイルス1型患者の発生： 2002年4月中旬，Townsville から約40km 南に在住する成人男性が EIA によりデング陽性との届け出があった。患者には最近の海外または Kuranda への旅行歴はなかったが，ウイルスへの推定曝露期間中に，単独で Townsville の Railway Estate を日帰りで訪れていた。1週間後，HI テストによりデング熱と確定し，デングウイルス1型によるものと判明した。調査により，Railway Estate に住む成人女性が4月上旬に発疹を伴なう軽度の熱性疾患を有していたことが明らかとなり，後に彼女はデング熱と確定された。

その後デング熱患者の発生はなく，デング1型患者の発生源は不明のままとなった。PCR 産物やデングウイルスは得られなかったため，分子生物学的解析は行えなかった。

Cairns におけるデングウイルス4型患者の発生： 2002年5月中旬，Cairns の北部郊外にある Smithfield に住む成人女性の血清から，PCR とウイルス分離の両方によりデングウイルス4型が同定されたとの届け出があった。患者はデング様症状を有しており，最近の Cairns から外部への旅行歴はなかった。しかし，彼女の発症以前に，彼女のパートナーの男性がインドネシ

ラジオたんぱ・アボット感染症アワー・放送内容集
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感染症と化学療法

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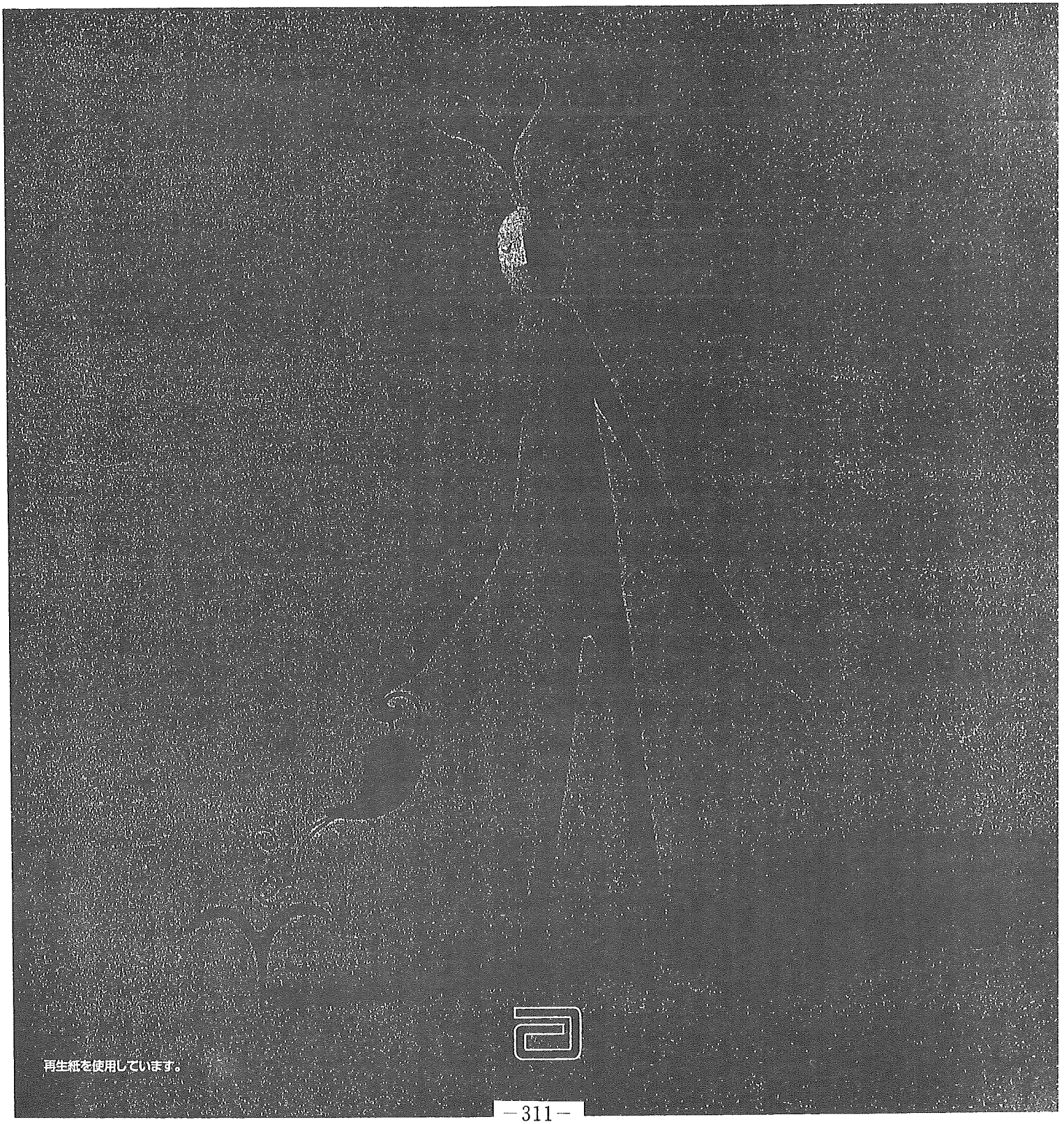
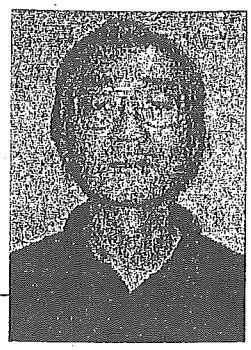


再生紙を使用しています。

マクロライド耐性肺炎マイコプラズマ感染症



札幌鉄道病院小児科　成田　光生 先生

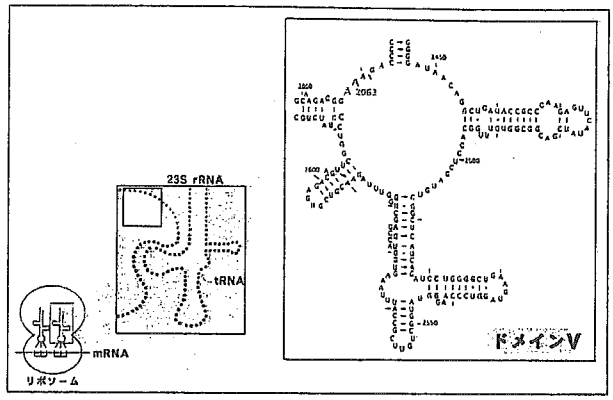
はじめに

マイコプラズマ肺炎の治療には一般的にマクロライド剤が第1選択として使用され，その他，テトラサイクリン系，リンコサミド系，さらに最近ではキノロン系の合成抗菌剤などが使用されていることは周知のとおりである．これはマイコプラズマは他の一般細菌とは異なり細菌壁をもたないため細胞壁合成阻害剤は機能せず，蛋白合成阻害作用をもつ薬剤が必要となるためである．この点について本稿の話題であるマクロライド耐性マイコプラズマを理解するためには，まず基本的に蛋白合成機構そのものを理解する必要があり，簡単に述べる．

マイコプラズマのマクロライド耐性獲得機序

蛋白は細胞内のリボソームで合成される．リボソームはリボソームRNAと20種類以上の蛋白質から構成されている．このうちマクロライド剤が作用するのは23Sリボソーム RNAのドメインVと呼ばれる部分であり（図1），マクロライド剤はここに結合することにより，その機能を阻害し，蛋白合成を抑制する．したがって，ドメインV遺伝子に変異が生じてマクロライド剤が結合できなくなると蛋白合成を阻害できず，抗生剤として機能しなくなる．

図1　マクロライド系薬剤の作用部位



当科で経験したマクロライド耐性株によるマイコプラズマ肺炎症例

我々は平成12年秋，札幌市において臨床的にクリンダマイシン耐性のマイコプラズマ肺炎症例の咽頭からマイコプラズマを分離し性状解析を行った[1]．その結果，その分離株は先述した薬剤耐性となる遺伝子変異を有する野生株であることが判明した．その後も日本各地から臨床的な印象から薬剤耐性とされたマイコプラズマ肺炎の症例が報告されている．本稿においては，まず札幌における最初のマクロライド耐性株による肺炎症例を紹介し，その後，マクロライド耐性マイコプラズマ肺炎について述べる．

❶患者背景・初発症状・診断

症例は9歳，女児で，平成12年11月25日から39℃台の発熱が出現し，28日，近医の内科を受診し胸部写真にて左中肺野の肺炎と診断された．

❷近医での治療経過・検査所見

外来にて7日間連日クリンダマイシンの点滴

静注を受けたが改善せず，12月5日には右上葉にも無気肺像が出現，入院となった．白血球数5900/mm³，CRP 2.12mg/dLと炎症反応は軽度であった．

❸当科における治療経過・検査所見

当初からクリンダマイシン耐性マイコプラズマ肺炎と考え，クラリスロマイシン単剤にて治療を開始，2日間で解熱し，両肺野の陰影も速やかに改善した．マイコプラズマ抗体価は320～10,240倍に上昇した．

❹薬剤感受性試験

1）方法

本患者の咽頭から自家製のPPLO培地を用いマイコプラズマの分離を行い，神奈川県衛生研究所に分解株を送付し薬剤感受性試験を施行した．マイコプラズマは増殖速度が極めて遅いため，最初に分離株を純培養に近い状態で得るまで約4週間を要し，さらに薬剤感受性試験そのものに約2週間を要するため，結果が出るまでには少なくとも6週間程度は必要である．したがって，この感受性試験は今後のための研究的あるいは疫学的な点で非常に重要な意味をもつが，通常の細菌検査とは異なり，残念ながら，その株が分離された患者自身の治療には間に合わないのである．

2）結果

検査の結果，本株はクリンダマイシンと同属のリンコマイシンのみならず，臨床的には著効を示したクラリスロマイシン，エリスロマイシンを含む14員環マクロライドに対しても高度の耐性を示した．また，ジョサマイシン，スピラマイシンなどの16員環マクロライドに対しても一定レベルの耐性を示したが，テトラサイクリンには感受性であった．

本例は筆者自身が治療した例であり，「クラリスロマイシンに変更後の急速な改善は単なる自然経過による治癒ということだけでは説明できない」という強い印象があったため，クラリスロマイシンにも耐性であったというのは意外な結果であった．実際に株が採取され，その性質が解析されていなければ，臨床的にはクリンダマイシンには耐性であっても，間違いなくクラリスロマイシンには感受性のマイコプラズマと判断されていたであろう．

❺遺伝子解析

1）方法

さらに，この株をいったん菌体を純培養にて増やした後，PCR法にてさらに必要領域を増幅し遺伝子解析を施行した．

2）結果

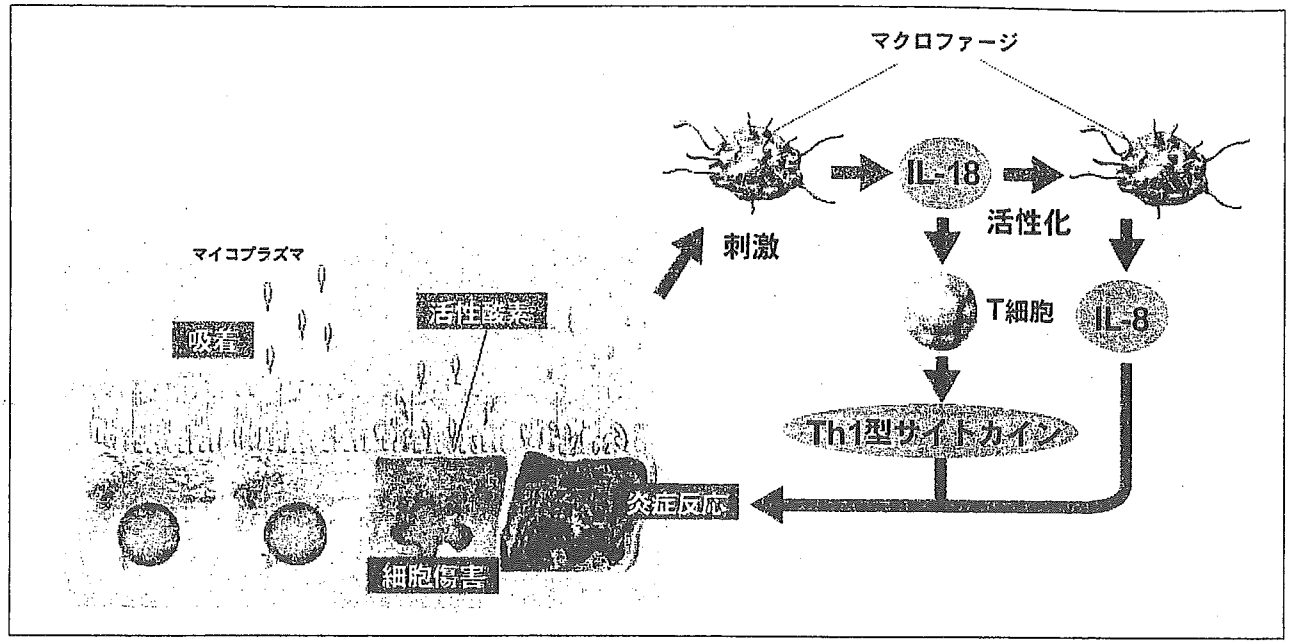
その結果，先述した23SリボソームRNAドメインVの2063番目の塩基にあたるアデニンがグアニンに置換しているという遺伝子変異が認められた．これにより23SリボソームRNAの立体構造に微妙な変化が生じ，マクロライド系薬剤はその作用点を失うため，マクロライドのなかでもとりわけ14員環および15員環に対して強い耐性が出現するのである．この2063番目のアデニンは，たとえば大腸菌では2058番目になるなど，厳密な数字は細菌の種類により異なるが，ここに相当する遺伝子変異により大腸菌，ブドウ球菌，肺炎球菌，*Helicobacter*など他の一般細菌においてもマクロライド系およびリンコサミド系の薬剤に耐性となることがよく知られているいわゆるホットスポットなのである[2]．

マクロライド耐性マイコプラズマ肺炎がマクロライド剤投与により臨床的に改善した理由

本例を含めた4株のマクロライド耐性マイコプラズマを分離したが，興味深いことに，4例すべてがマクロライド剤投与により臨床的には改善したのである．その理由について述べる．

現在までの研究ではマイコプラズマ自体には活性酸素を産生して呼吸器粘膜を障害する程度の能力しかなく，マイコプラズマ感染症における肺炎の病像は宿主の免疫応答により形成されているということは一般に認められている．この点に関し，マイコプラズマ肺炎の病変形成においてはマクロファージが起点となり，一方でTh1型サイトカイン産生の亢進により炎症が惹起される．もう一方ではIL-18を介したIL-8産生が重要な役割を演じている可能性がある（図2）．

図2　マイコプラズマ肺炎の発症機構



そこで注目されるのがマクロライドの新作用である．14員環及び15員環マクロライドには気管支上皮細胞からのこれらのサイトカイン産生を抑制する作用があることが報告されている．

以上のようなことから，いまだ仮説の段階ではあるが，マイコプラズマ肺炎に対する14員環及び15員環マクロライドの治療効果には抗菌作用と免疫修飾作用の二面性があるのではないかと考えられるのである．すなわち，病初期の感染成立から増殖過程では当然ながら抗生剤としての抗菌作用が主体であり，ここでは薬剤感受性が重要な問題となる．この段階で，もし感染した株がマクロライド耐性の場合には，抗生剤としての臨床効果は発揮されないと考えられる．その後，宿主の免疫機構が立ち上がってくると，その免疫機能によりマイコプラズマ自体の排除が開始されるものの，その一方で肺炎の病像が形成されてくる．この段階では抗菌作用よりも免疫修飾作用が治療効果としての主体となっているとすれば，マクロライド耐性マイコプラズマ肺炎において分離株が*in vitro*でマクロライド耐性であったにもかかわらず臨床的に治療効果が認められた現象の説明が可能であると考える．

マクロライド耐性株による マイコプラズマ肺炎の問題点

筆者の得た実際のevidenceをもとにマクロライド耐性株によるマイコプラズマ肺炎の問題点をまとめる．

1) マクロライド耐性マイコプラズマは確かに野生に存在するが，耐性株は決して感染力も強いというわけではなく，たまたま1例，そのような症例が出たとしても，それがそのまま耐性株主体の流行となる傾向は現在のところ認められていない．
2) マクロライド耐性のメカニズムに関しては，マイコプラズマで実際に証明されたのはドメインⅤの点変異のみで，メチル化遺伝子や薬剤排出ポンプの存在は確認されていない．
3) 抗生剤に対する反応に基づく臨床的印象と実際の分離株における耐性の有無は必ずしも一致しない．マイコプラズマ肺炎に対するマクロライド剤の臨床効果が抗生剤としての作用のみではない可能性が示唆され，今後の検討課題であると考えられる．

おわりに

マクロライド耐性マイコプラズマの研究は神奈川県衛生研究所の岡崎則男先生および国立感染症研究所の佐々木次雄先生を中心とする研究室の方々との共同研究であり，謝意を表したい．

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