Table 1. *Distribution of MICs for various antibiotics for 114 isolates of pneumococci in Japan*

	No. of isolates for which MIC (μg/ml)															
Antibiotic	⩽0·004	0·008	0·016	0·032	0·063	0·125	0·25	0·5	1	2	4	8	16	32	64	⩾128
Penicillin G		1	3	26	18	11	9	5	15 ⇓	25	1					
Ceftriaxone			4	6	1	22	25	23	33							
Cefditoren		1	3	9	4	26	18	40	10	3 ⇓						
Imipenem	9	36	15	6	5	14	28	⇓	1							
Erythromycin				3	10	7	2	6 ⇓	13	10	3	3	1			56
Clarithromycin			1	6	11	7		8	12 ⇓	9	2		2			56
Azithromycin				2		14	6	8	14	3 ⇓	5		2		1	59
Levofloxacin								3	50	59	1 ⇓			1		
Vancomycin						1	93	20								

⇓ Breakpoints for each antibiotic.

difference in profile needed to distinguish between PFGE types.

RESULTS

Antimicrobial susceptibility test

The MICs of the nine antibiotics tested for the 114 *S. pneumoniae* isolates are shown in Table 1. Using NCCLS breakpoints all isolates were found to be susceptible to ceftriaxone, imipenem and vancomycin. The majority were resistant to erythromycin, clarithromycin and azithromycin; only two isolates were not susceptible to levofloxacin. Forty-eight isolates (42·1%) were susceptible to penicillin G, 40 (35·1%) showed intermediate resistance and 26 (22·8%) were fully resistant to ⩾2·0 μg/ml of this antibiotic.

Serotyping

All isolates but one, were grouped into 21 different serotypes (Table 2). The serotypes of penicillin-susceptible isolates varied widely but with serotypes 3 (22·9%), 6B (12·5%) and 19F (12·5%) predominating among 18 other serotypes. In contrast, the 26 fully penicillin-resistant isolates fell into only six serotypes, the most frequent being 19F (50·0%), and 23F (23·1%); intermediate susceptible isolates were represented by 11 serotypes with 23F (20·0%), 14 (17·5%), 6B (15·0%) and 19F (15·0%) being the most common.

Molecular characterization of serotypes 19F and 23F

PFGE typing revealed 10 different DNA profiles (A–J) among the 25 strains of serotype 19F (Fig. 1). One pattern, type A, accounted for 14 strains and this was indistinguishable from the profile of the Taiwan multidrug-resistant serotype 19F clone. Three strains of serotype 19F were classified as pattern B and the other eight strains gave unique patterns. Twelve of the pattern A strains were not susceptible to penicillin and all 14 harboured the *mef*(A) gene. Overall 21 (84%) of the serotype 19F strains were *mef*(A) and/or *erm*(B) positive. Eighteen of the serotype 19F strains had alterations in *pbp1a*, *−2x*, and *−2b*; four in *pbp2x* and *−2b*; two in *pbp2x*; and one had no alteration. These strains originated from different geographical regions of Japan (Table 3).

The 15 strains of serotype 23F were characterized by nine different DNA profiles (b–j) with pattern b predominating and represented by six strains (Fig. 2). Two strains were typed as pattern g and the remainder were unique. None of the DNA profiles of serotype 23F matched that of the Spanish multidrug-resistant 23F clone but pattern b was similar to the profile of the Taiwan multidrug-resistant serotype

Table 2. *Serotypes of susceptible, intermediate, and fully penicillin-resistant pneumococci*

Serotype	Susceptible ($n=48$)	Intermediate ($n=40$)	Resistant ($n=26$)
3	11	2	0
6A	3	1	3
6B	6	6	2
7F	1	0	0
9A	1	0	0
9V	4	0	1
11A	2	2	1
14	2	7	0
15A	1	0	0
15C	2	0	0
16F	0	3	0
18A	1	0	0
18B	1	0	0
18C	1	0	0
19A	3	2	0
19F	6	6	13
22F	1	0	0
23A	0	1	0
23F	1	8	6
29	0	1	0
34	1	0	0
Non-typable	0	1	0

23F clone although there were at least four band differences between them (Fig. 2). Strains of pattern b were not susceptible to penicillin and all positive by PCR for either the *mef*(A) or *erm*(B) gene as were strains of the other DNA types. These strains were also widespread in the country (Table 3). Thirteen of the serotype 23F strains were altered in *pbp1a*, *−2x*, and *−2b*; and one each in *pbp2x* and *−2b*, and *pbp2x* respectively.

Two strains each of serotype 19F (patterns A2 and B) and 23F (patterns e and i) had both *mef*(A) and *erm*(B) genes, and these four strains were all penicillin non-susceptible (Table 3).

DISCUSSION

Penicillin-resistant *S. pneumoniae* are already distributed worldwide and resistance appears to be expanding to include multiple antimicrobial agents [1, 4]. In Asia, the proportion of *S. pneumoniae* reported to be non-susceptible to penicillin over the last decade ranges from 68·8% in Thailand [12], to 3·8% in India, 9·0% in Malaysia, 9·8% in China, 21·0% in Indonesia, 23·1% in Singapore, 38·7% in Taiwan, 41·2% in Sri Lanka, 60·8% in Vietnam, 79·7% in

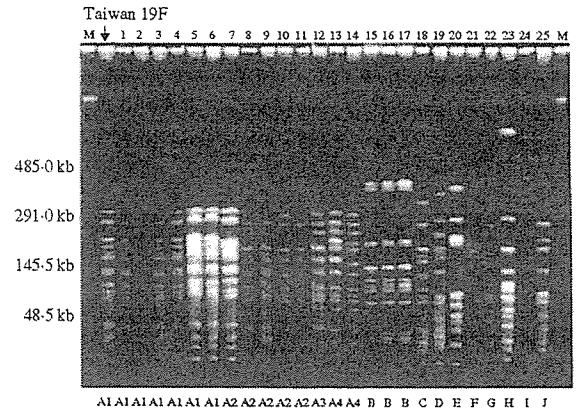

Fig. 1. Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested DNA from 25 strains of serotype 19F *S. pneumoniae* and a representative of the Taiwan multi-resistant serotype 19F clone. M, Molecular size marker.

Korea and 65·3% in Japan in 1996 to 1997 [13]. Indeed, a survey of a single prefecture in Japan from 2001 to 2003 reported a frequency of 60·9% [6]. In this study, 57·9% of the *S. pneumoniae* isolates from patients with CAP were penicillin non-susceptible and some of these were also resistant to various other antibiotics. Thus, it appears that the frequency of multidrug-resistant *S. pneumoniae* in Japan is among the highest in Asia. A previous study demonstrated that the aetiology of CAP in Japan did not differ substantially from Western countries and that *S. pneumoniae* was the most common pathogen in this disease [3]. Some earlier surveys indicate that the clinical outcome between penicillin-susceptible and non-susceptible isolates does not differ under current resistance levels [5, 14]. However, invasive pneumococcal isolates with very high-level penicillin resistance (MIC $\geqslant 8{\cdot}0\ \mu g/ml$) have been reported to be prevalent in the United States [15]. Caution should be exercised concerning such strains, although we did not detect them in Japan.

The 23-valent pneumococcal polysaccharide vaccine (PS23) has been promoted worldwide, although it fails to protect children <2 years old [16, 17]. It is well known that children aged <5 years and adults of $\geqslant 65$ years are the most susceptible to invasive pneumococcal infection and have a high mortality rate [16, 18]. Vaccination, therefore, seems to be the only available method for preventing pneumococcal disease [16, 19]. In this study, all the isolates were classified into 21 serotypes, 82·5% of which are accounted for in PS23. Furthermore, 56 (59·6%) of the isolates were penicillin non-susceptible isolates.

Table 3. *Correlation of PFGE patterns, penicillin resistance, macrolide resistance genes and mutation in penicillin binding protein genes in strains of* S. pneumoniae *serotypes 19F and 23F*

PFGE line	PFGE pattern	Penicillin resistant	autolysin	*pbp1a*	*pbp2x*	*pbp2b*	*mef*(A)	*erm*(B)	Region
19F									
1	A1	R	+	+	+	+	+	−	Tohoku
2	A1	S	+	−	+	−	+	−	Kyushu
3	A1	S	+	−	+	−	+	−	Kyushu
4	A1	R	+	+	+	+	+	−	Kinki
5	A1	R	+	+	+	+	+	−	Kanto
6	A1	R	+	+	+	+	+	−	Kinki
7	A2	R	+	+	+	+	+	+	Kyushu
8	A2	I	+	+	+	+	+	−	Chubu
9	A2	R	+	+	+	+	+	−	Kanto
10	A2	I	+	+	+	+	+	−	Kanto
11	A2	R	+	+	+	+	+	−	Kanto
12	A3	R	+	+	+	+	+	−	Chubu
13	A4	R	+	+	+	+	+	−	Kinki
14	A4	I	+	+	+	+	+	−	Kanto
15	B	I	+	+	+	+	+	−	Kanto
16	B	R	+	+	+	+	+	+	Kanto
17	B	S	+	−	+	+	−	+	Kyushu
18	C	I	+	+	+	+	+	−	Kanto
19	D	R	+	+	+	+	+	−	Kinki
20	E	I	+	−	+	+	−	+	Kanto
21	F	R	+	+	+	+	−	+	Kanto
22	G	S	+	−	+	+	−	−	Kanto
23	H	S	+	−	+	+	−	−	Chugoku
24	I	S	+	−	−	−	−	−	Kanto
25	J	R	+	+	+	+	−	−	Kanto
23F									
1	b2	R	+	+	+	+	−	+	Kinki
2	b3	I	+	+	+	+	+	−	Kanto
3	b2	I	+	+	+	+	+	−	Kanto
4	b2	I	+	+	+	+	−	+	Kanto
5	b3	I	+	+	+	+	−	+	Chuguku
6	b3	I	+	+	+	+	+	−	Kanto
7	c	I	+	−	+	+	−	−	Kyushu
8	d	R	+	+	+	+	−	+	Kanto
9	e	R	+	+	+	+	+	+	Kanto
10	f	I	+	+	+	+	−	+	Kanto
11	g	S	+	−	+	−	−	+	Kanto
12	g	R	+	+	+	+	−	+	Kyushu
13	h	R	+	+	+	+	+	−	Kanto
14	i	I	+	+	+	+	+	+	Kinki
15	j	R	+	+	+	+	−	+	Kanto

S, penicillin-susceptible. I, penicillin intermediate resistant. R, penicillin-resistant.
Penicillin binding protein genes *pbp1a*, *pbp2x*, and *pbp2a*: +, altered; −, not altered.
Macrolide resistant genes *mef*(A) and *erm*(B): +, positive; −, negative.

Although the efficacy of PS23 against pneumonia without bacteraemia is controversial [20], a combination of influenza and pneumococcal vaccination appears to be beneficial for the reduction of mortality from all causes in individuals aged ⩾65 years [21].

In the group of serotype 23F pneumococci, six out of 15 strains showed the predominant PFGE pattern b which was different from the pattern of the Spanish multidrug-resistant 23F clone but similar to the Taiwan multidrug-resistant serotype 23F clone.

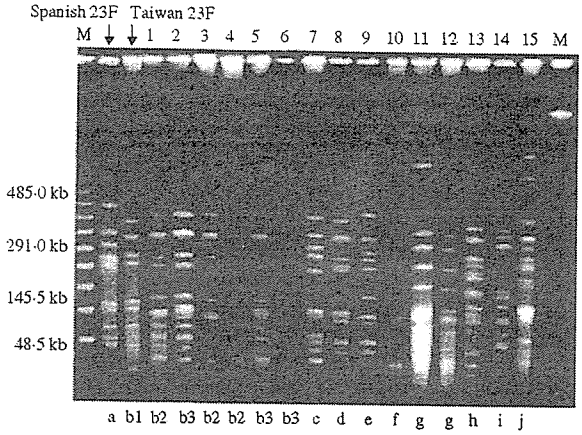

Fig. 2. Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested DNA from 15 strains of serotype 23F *S. pneumoniae* and representatives of the Spanish and Taiwan multiresistant serotype 23F clone. M, Molecular size marker.

Although a previous study indicated that the Spanish multidrug-resistant 23F clone was spreading in Asia [13], it was not detected in this survey. On the other hand, 14 out of 25 strains of serotype 19F showed the predominant pattern A which was closely related to the pattern for the Taiwan multidrug-resistant 19F clone. Since these predominant serotypes were collected from different regions in Japan and most were penicillin non-susceptible with multiple alterations in *pbp* and all isolates had macrolide resistant genes, these results suggest that derivatives of the Taiwan multidrug-resistant 19F and 23F clones have the potential to spread further in Japan. Recently, it was reported by Kasahara *et al.* [6] that these clones had already spread in Japan. However, theirs was a pilot study and they recognized the need for an urgent nationwide surveillance for these strains. Our nationwide study focusing on CAP-associated pneumococci has yielded results consistent with their earlier findings.

Moreover, it has been suggested in a recent report that pneumococcal isolates containing both the *mef*(A) and *erm*(B) genes may have originated from the Taiwan multidrug-resistant serotype 19F clone containing the *mef*(A) gene after introduction of the *erm*(B) gene [22]. However, one serotype 19F strain different from the Taiwan serotype 19F clone and two serotype 23F strains containing both the *mef*(A) and *erm*(B) genes were identified in our study suggesting that other types of antibiotic-resistant pneumococci might appear in Japan.

In conclusion, our results indicate that multidrug-resistant pneumococci are spreading rapidly in Japan and that efforts to prevent spread of pandemic multidrug-resistant serotype 19F and 23F clones should be intensified.

ACKNOWLEDGEMENTS

We thank all the staff in participating institutions of this study: Department of Internal Medicine, Graduate School of Medicine, Tohoku University (5 patients), Shiogama Municipal Hospital (2 patients), Department of Respirology, Graduate School of Medicine, Chiba University (4 patients), Funabashi Municipal Medical Center (1 patient), Chiba Municipal Kaihin Hospital (1 patient), Department of Respiratory Medicine, Juntendo University School of Medicine (5 patients), Fourth Department of Internal Medicine, Nippon Medical School (4 patients), First Department of Internal Medicine, Kyorin University, School of Medicine (26 patients), National Organization Tokyo Hospital (20 patients), National Center for Geriatrics and Gerontology (2 patients), Nagoya Ekisaikai Hospital (1 patient), Anjo Kosei Hospital (3 patients), Aichi Prefectural Showa Hospital (3 patients), First Department of Internal Medicine, Osaka Medical College (8 patients), Minoh Municipal Hospital (6 patients), Department of Respiratory Medicine, Kawasaki Medical University (3 patients), Kyorin Hospital (7 patients), Tagami Hospital (2 patients), Nagasaki Medical Center of Neurology (9 patients), Takasugi Clinic (2 patients). We also thank Banyu Pharmaceutical Co., Tokyo, Japan for help in completing this study. We also thank Akihiro Wada (Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University), Chieko Shimauchi (Miyazaki Prefectural Nursing University) and Matsuhisa Inoue (Kitasato University School of Medicine) for their help in completing the PFGE studies. Thanks are also due to Kei Kasahara (Second Department of Internal Medicine, Nara Medical University) for providing the Taiwan multidrug-resistant serotype 23F clone.

This study was supported by a Grant-in-Aid for U.S.–Japan Cooperative Medical Science Program, Acute Respiratory Infections Panel.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Appelbaum PC.** Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clinical Infectious Diseases* 1992; **15**: 77–83.
2. **Linares J, *et al.*** Trends in antimicrobial resistance of clinical isolates of *Streptococcus pneumoniae* in Bellvitge Hospital, Barcelona, Spain (1979–1990). *Clinical Infectious Diseases* 1992; **15**: 99–105.
3. **Ishida T, *et al.*** Etiology of community-acquired pneumonia in hospitalized patients: a 3-year prospective study in Japan. *Chest* 1998; **114**: 1588–1593.
4. **Rikitomi N, *et al.*** Rapid increase of pneumococcal resistance to β-lactam and other antibiotics in isolates from the respiratory tract (Nagasaki, Japan: 1975–1994). *Microbiology and Immunology* 1996; **40**: 899–905.
5. **Watanabe H, *et al.*** A comparative clinical study of pneumonia by penicillin-resistant and -sensitive *Streptococcus pneumoniae* in a community hospital. *Respirology* 2000; **5**: 59–64.
6. **Kasahara K, *et al.*** Clonal dissemination of macrolide-resistant and penicillin-susceptible serotype 3 and penicillin-resistant Taiwan 19F-14 and 23F-15 *Streptococcus pneumoniae* isolates in Japan: a pilot surveillance study. *Journal of Clinical Microbiology* 2005; **43**: 1640–1645.
7. **NCCLS.** Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A4. National Committee for Clinical Laboratory Standards, Wayne, PA, 1998.
8. **Ubukata K, Iwata S, Sunakawa K.** *In vitro* activities of new ketolide, telithromycin, and eight other macrolide antibiotics against *Streptococcus pneumoniae* having mefA and ermB genes that mediate macrolide resistance. *Journal of Infection and Chemotherapy* 2003; **9**: 221–226.
9. **McGee L, *et al.*** Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the Pneumococcal Molecular Epidemiology Network. *Journal of Clinical Microbiology* 2001; **39**: 2565–2571.
10. **Yano H, *et al.*** Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. *Journal of Clinical Microbiology* 2000; **38**: 625–629.
11. **Tenover FC, *et al.*** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* 1995; **33**: 2233–2239.
12. **Watanabe H, *et al.*** Antimicrobial susceptibility and serotype distribution of *Streptococcus pneumoniae* and molecular characterization of multidrug-resistant serotype 19F, 6B, and 23F pneumococci in North Thailand. *Journal of Clinical Microbiology* 2003; **41**: 4178–4183.
13. **Song JH, *et al.*** Spread of drug-resistant *Streptococcus pneumoniae* in Asian countries: Asian Network for Surveillance of Resistant Pathogens (ANSORP) study. *Clinical Infectious Diseases* 1999; **28**: 1206–1211.
14. **Aspa J, *et al.*** Drug-resistant pneumococcal pneumonia: clinical relevance and related factors. *Clinical Infectious Diseases* 2004; **38**: 787–798.
15. **Schrag SJ, *et al.*** Emergence of *Streptococcus pneumoniae* with very-high-level resistance to penicillin. *Antimicrobial Agents and Chemotherapy* 2004; **48**: 3016–3023.
16. **Bogaert D, *et al.*** Pneumococcal vaccines: an update on current strategies. *Vaccine* 2004; **22**: 2209–2220.
17. **U.S. Centers for Disease Control.** Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morbidity and Mortality Weekly Report* 1997; **46 (RR-8)**: 1–24.
18. **Mantese OC, *et al.*** Prevalence of serotypes and antimicrobial resistance of invasive strains of *Streptococcus pneumoniae*. *Journal of Pediatrics* 2003; **79**: 537–542.
19. **Martens P, *et al.*** Serotype-specific mortality from invasive *Streptococcus pneumoniae* disease revisited. *BMC Infectious Diseases* 2004; **4**: 21.
20. **Mangtani P, Cutts F, Hall AJ.** Efficacy of polysaccharide pneumococcal vaccine in adults in more developed countries: the state of the evidence. *Lancet Infectious Diseases* 2003; **3**: 71–78.
21. **Christenson B, *et al.*** Effects of a large-scale intervention with influenza and 23-valent pneumococcal vaccines in adults aged 65 years or older: a prospective study. *Lancet* 2001; **357**: 1008–1011.
22. **Ko KS, Song JH.** Evolution of erythromycin-resistant *Streptococcus pneumoniae* from Asian countries that contains *erm*(B) and *mef*(A) genes. *Journal of Infectious Diseases* 2004; **190**: 739–747.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 24 (2006) 5896–5903
Vaccine
www.elsevier.com/locate/vaccine

Dynamics of dendritic cell migration and the subsequent induction of protective immunity in the lung after repeated airway challenges by nontypeable *Haemophilus influenzae* outer membrane protein

Shin-ichi Kurita [a,1], Jun Koyama [a,1], Shozaburo Onizuka [a], Kazushi Motomura [a], Hiroshi Watanabe [a], Kiwao Watanabe [a], Masachika Senba [b], Michael A. Apicella [e], Timothy F. Murphy [f], Horoyuki Yoneyama [d], Kouji Matsushima [d], Tsuyoshi Nagatake [a], Kazunori Oishi [a,c,*]

[a] *Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan*
[b] *Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan*
[c] *Department of Special Pathogen, Research Institute of Microbial Diseases, Osaka University, Suita, Japan*
[d] *Molecular Preventive Medicine, University of Tokyo School of Medicine, Bunkyoku, Tokyo, Japan*
[e] *Department of Microbiology, University of Iowa, Iowa City, Iowa, Japan*
[f] *Department of Medicine, State University of New York at Buffalo, USA*

Received 15 March 2005; received in revised form 20 June 2005; accepted 13 April 2006
Available online 19 May 2006

Abstract

To determine the dynamics of dendritic cell (DCs) migration and their role in recurrent infections by nontypeable *Haemophilus influenzae* (NTHi), the migration of mature DC into pulmonary lymph nodes (LN) and the development of a P6-specific immune response and bacterial clearance in the lung were examined after repeated airway challenges with outer membrane protein (OMP) at 1-week intervals in mice. Although the migration of mature DC into the pulmonary LN is attenuated after repeated airway challenge with OMP, the in vitro P6-specific T cell proliferation in the cultured pulmonary LN cells was enhanced and was subsequently linked to the development of P6-specific IgA production and the development of protective immunity in the airway of mice.

Keywords: Nontypeable *Haemophilus influenzae*; P6 protein; Dendritic cell

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of acute otitis media in children, and is also frequently associated with the acute exacerbation of chronic pulmonary diseases (COPD) [1,2]. An increasing body of evidence is available that indicates a high rate of antibiotic resistance of NTHi in such diseases [3,4]. A vaccinating strategy against NTHi infections is, therefore, urgently required for these populations [5]. P6 is a 16 kDa peptidoglycan-associated lipoprotein that is commonly found in the outer membrane of all strains of NTHi and exhibits a high degree of sequence conservation among strains [6,7]. Since mucosal immunization with P6 enhances the bacterial clearance of NTHi in the airway and the middle ear, this protein is considered to be viable candidate for use in a mucosal vaccine [8–12].

Dendritic cells (DCs) are antigen presenting cells that have a unique ability to induce primary immune responses, thus allowing the establishment of immunological memories

* Correspondence to: 3-1 Yamadaoka, Suita 565-0871, Japan. Tel.: +81 6 6879 4253; fax: +81 6 6879 4255.
E-mail address: oishik@biken.osaka-u.ac.jp (K. Oishi).
[1] S. Kurita and J. Koyama equally contributed to the work described in this paper.

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.04.041

[13,14]. After capturing antigens, DCs mature and express high levels of MHC class II and costimulatory molecules such as CD86 on their surfaces [15,16]. A previous study reported that DC precursors rapidly emerged into the airway epithelium in advance of neutrophil influx after the inhalation of *Moraxella catarrhalis* [17]. These cells are thought to differentiate into mature DC in the airway epithelium and migrate via the lymphatic system to regional lymph nodes through CC chemokine receptors (CCR7)/CCL19 (Epstein-Barr virus-induced molecule-1 ligand chemokine: ELC) or CCL21 (secondary lymphoid tissue chemokine; SLC) interactions [18–20].

Although recurrent infections due to NTHi are typically observed in patients with COPD, the dynamics of DC migration into the regional lymph nodes (LNs) and the subsequent induction of specific adaptive immunity after a recurrent infection with NTHi have not been fully investigated. In this study, the relationship between DC–T cell interaction in regional LNs upon an P6 and protective immunity in the airway after repeated airway challenges with outer membrane protein (OMP) of NTHi was examined in a murine model.

2. Materials and methods

2.1. Mice

Specific pathogen-free, 6–9-week-old female BALB/c mice were purchased from Charles River Japan, Kanagawa, Japan. The mice were maintained in barrier-protected animal facilities under specific pathogen-free conditions using ventilated microisolator cages in the experimental animal facility of the Institute of Tropical Medicine, Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University and conformed to National Institute of Health guidelines.

2.2. OMP preparation

A clinical isolate of NTHi (H93-151) from a patient with a respiratory tract infection was obtained and stored at −80 °C. OMP was prepared by a previously described method, with minor modifications [6]. In a typical experiment, NTHi was cultured overnight on brain heart infusion (BHI) agar (BBL, Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) containing 5% Fildes enrichment (Becton Dickinson and Co.) and 1% isovital X (Becton Dickinson and Co.) at 37 °C in a CO_2 incubator. The bacteria were harvested, suspended in 10 mM HEPES (Sigma Chemical Co., St. Louis, MO) buffer (pH 7.4), and disrupted by sonication. Debris was removed by centrifugation at 1700 × *g* for 20 min. The supernatants were pooled and centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet was suspended in a solution containing equal volumes of 10 mM HEPES buffer (pH 7.4) and 2% aqueous sodium lauryl sarcosinate (Wako Pure Chemical Industries, Osaka, Japan). The suspension was then centrifuged at 100,000 × *g* for 1 h min at 4 °C. The pellet was suspended in distilled water and lyophilized. The resulting powder was used as OMP, and stored until used.

2.3. Intratracheal challenge of OMP

Twenty micrograms of OMP from an NTHi strain (H93-151) in a volume of 25 μl was intratracheally administered to each mouse on days 0, 7 and 14. Day 0 was defined as the day of the initial intratracheal (IT) challenge. The procedures were performed under anesthesia with an intraperitoneal injection of 0.2 ml of a solution containing 3 mg of ketamine and 0.1 mg of xylazine. The day 7 was defined as immediately prior to the second IT challenge, and the day 14 was immediately before the third challenge, respectively. Heparinized blood was obtained, the plasma separated, and BAL was performed at the indicated times after the initial challenge, as previously described [21]. Plasma and BAL fluid were stored at −80 °C until used.

2.4. Cell preparation and flow cytometry

A single cell suspension was obtained from pulmonary LNs of mice that had received the IT challenges of OMP or untreated mice as described previously [16]. The cells were incubated in RPMI-1640 medium containing 1 mg/ml collagenase type I (Wako Pure Chemistry) and 40 μg/ml DNase I (Roche, Mannheim, Germany) for 30 min at 37 °C. Pulmonary LN cells were subjected to FACS analysis (FACSCalibur, BD Biosciences) and the data were analyzed using the Cell Quest software program. These cells were stained with PE-conjugated rat anti-mouse CD11c monoclonal antibody (mAb) (BD PharMingen, San Diego, CA) and FITC-conjugated hamster anti-mouse CD86 mAb (BD PharMingen). The cultured pulmonary LN cells were stained with FITC-conjugated anti-mouse CD3 (BD Phramingen) or PE-conjugated anti-mouse B220 mAb (BD Phramingen).

2.5. Cell proliferation assay

In vitro cell proliferation assays were carried out using the Prenix WST-1 cell proliferation assay system (TAKARA, Tokyo, Japan) according to the manufacturer's instructions. Mononuclear cells were prepared from the pulmonary LNs of mice that had received an IT challenge of OMP once, twice or three times at 1-week intervals (days 0, 7 and 14) as described previously [16]. LN cells at a concentration of 1×10^6 in 100 μl of RPMI medium containing 10% FCS were seeded in a 96-well chamber in triplicate and were stimulated at 37 °C for 72 h with or without antigens at concentrations of 5 or 10 μg/ml. Purified P6 protein, ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) and lipooligosaccharide (LOS) from NTHi were used as antigens. The P6 protein was purified, as described previously [6]. The LOS from NTHi was also purified as described previously [22]. After incubation,

the assay was developed by the addition of a solution of Premix WST-1 (10 μg/well) to each well. The optical density (OD) was measured at 450 and 650 nm after a 4 h incubation at 37 °C. The data were calculated by subtracting the OD_{650} value from the OD_{450} value.

2.6. P6-specific antibodies by ELISA

The P6-specific antibody titer in plasma or BAL fluid was determined by ELISA according to a previously published method [23]. An antigen preparation of P6 was used as the coating antigen (1 μg/ml). The plate was washed, and then reacted with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgM or IgG or IgA (Zymed, San Francisco, CA). The OD at 405 nm was then measured. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution giving an OD_{450} of $\geq$0.1 OD unit above the OD_{450} of negative control samples obtained from non-immunized mice.

2.7. P6-specific antibody-forming cells (AFC) by ELISPOT

Individual wells of a 96-well multiscreen filterplate (Millipore, Bedford, MA) were coated overnight with 1 μg/ml of P6 protein in phosphate-buffered saline (PBS). The plates were incubated overnight at 4 °C and washed the following day with PBS. Nonspecific binding was blocked by treatment with 1% BSA in PBS. Single cell suspensions from the spleens of non-immunized or immunized mice were prepared in RPMI-1640 medium (starting at 1×10^6 cells/well) and were added to the appropriate wells. After incubation for 4 h at 37 °C, the cells were removed from the plate and the wells were washed three times. The plates were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgM, IgG or IgA (Southern Biotechnology Associates Inc, Birmingham, Al) overnight at 4 °C and washed again with PBS [24]. Spot-forming cells were detected by the addition of 1.6 mM of 3-amino-9-ethylcarbazole (Sigma) in 50 mM sodium acetate buffer (pH 5.0) containing 0.05% H_2O_2. The plates were washed with water, dried, and the number of P6-specific AFCs was quantified under a stereomicroscope.

2.8. Lung histopathology

For histochemical examination, fresh lung tissue was fixed in 4% paraformaldehyde and embedded with paraffin for hematoxylin and eosin staining and the sections were then examined by light microscopy. For the immunohistochemical examination, the tissue was embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN, USA) at −80 °C, cut with cryostat at 6 μm and acetone-fixed fresh frozen tissue sections were immunostained with biotinylated anti-mouse IgA (BD Bioscience, Franklin Lakes, NJ), followed by avidin conjugated Alexa 488 (Molecular Probe Inc.). The sections were observed by fluorescence microscopy.

2.9. Bacterial clearance

NTHi (H93-151) strain at a dose of 1×10^8 cfu/head was intranasally challenged to mice that had previously received an IT challenge of NTHi OMP or sterile PBS, three times at 1-week intervals, or untreated mice. Quantitative bacterial cultures of lung tissue from mice that had been euthanized with pentobarbital were performed on agar 1, 4 and 8 h after the bacterial challenge. The lungs were removed aseptically and homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing. The detection limit of bacterial culture of the lung tissue was 10^3 cfu/g.

2.10. Statistical analysis

Statistical analyses were performed by one-way ANOVA and multiple comparison methods by Bonferroni-Dunn's test or using unpaired Student's *t*-test. Data were considered to be statistically significant, if the *P* values were less than 0.05. All data were expressed as mean ± S.D.

3. Results

3.1. Influx of mature DC in the pulmonary lymph nodes

After the initial intratracheal challenge of NTHi OMP, a rapid increase in neutrophil levels was detected in BAL fluids. The number of neutrophils in the BAL fluids peaked at day 2, and then gradually declined until day 7 post-challenge (data not shown). A rapid increase in the absolute number of $CD11c^+/CD86^+$ cells was found in the pulmonary LN after the IT challenge of NTHi OMP (Fig. 1). These mature DC peaked at day 1 and then gradually declined until day 7. After the repeated IT challenge of OMP at 1-week intervals, the levels of neutrophils that accumulated in the BAL fluid similarly peaked 1 day after the challenge. A gradual increase in the numbers of alveolar macrophages (AM) and lymphocytes was noted after the repeated IT challenge (data not shown). Following the rapid accumulation of neutrophils in BAL fluid after the second (day 7) and third challenge (day 14), an additional influx of $CD11c^+/CD86^+$ cells was found in the pulmonary LN. The absolute number of DCs 1 day after the second IT challenge or the third IT challenge tended to be lower than that of DCs in the pulmonary LN 1 day after the initial challenge, although no statistical significance was found between them.

3.2. P6-specific proliferation of pulmonary LN cells

No cell proliferation was found in cultured cells isolated from the pulmonary LN of untreated mice in the presence of P6 (data not shown). The cultured cells isolated from mice that had received a single IT challenges, two serial IT challenges of OMP or serial three IT challenges at 1-week intervals similarly showed no proliferation in the presence

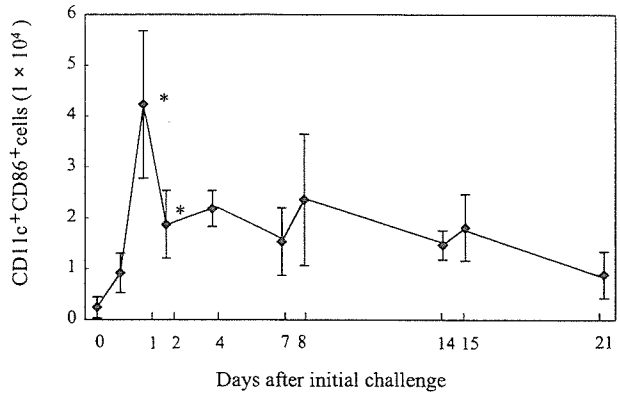

Fig. 1. Kinetic cell analysis of the absolute number of mature dendritic cells (DCs) in pulmonary lymph node (LN) cells in mice before and after repeated intratracheal (IT) challenges of outer membrane protein (OMP). The mice received an IT challenge of OMP at days 0, 7 and 14. LN cells were prepared from pulmonary LNs before the challenge and at 6 h, 12 h, day 1, day 2, day 4 and day 7 before and the second challenge (at day 7), and at days 8 and 14 before the third challenge (at day 14) and at day 21 after the initial IT challenge. These cells were stained for CD11c and CD86, and subjected to FACS analysis, and the percentages of $CD11c^+/CD86^+$ cells are shown. The numbers of $CD11c^+/CD86^+$ cells from the pulmonary LNs are indicated by counting the numbers of LN cells and gating on the monocytes according to forward and side scatter. $^*P<0.01$ (versus the number of DCs at day 0). Values represent mean ± S.D. of four animals.

of ovalbumin (OVA) (Fig. 2A). No cell proliferation was found in the cultured cells isolated from pulmonary LN cells of mice that received three serial IT challenges of OMP in the presence of lipooligosaccharide (LOS) from NTHi (data not shown). In contrast, a dose-dependent increase in cell proliferation was found in cultured cells isolated from the pulmonary LN of mice that had received repeated IT challenges in the presence of P6 at 5 and 10 μg/ml ($P<0.01$ or $P<0.05$). A significant increase in cell proliferation was also found in the presence of P6 at 5 and 10 μg/ml ($P<0.01$), but the cell proliferation declined in the presence of P6 at 10 μg/ml in the cultured cells isolated from mice that received a single IT challenge of OMP. The ratio of T cells to B cells in the proliferating cells in the presence of P6 at 5 μg/ml was significantly higher than that in the presence of OVA at the same concentration ($P<0.01$). Collectively, the above data suggest that the P6-specific T cell proliferation occurred in the pulmonary LN after a single or repeated IT challenges of OMP, although the absolute number of mature DC that migrated into the pulmonary LN declined after repeated IT challenges.

3.3. P6-specific Igs and AFC

An increase in P6-specific IgM forming cells in the spleen was found to be associated with the appearance of P6-specific IgM in the plasma at day 7 (Figs. 3 and 4A). While P6-specific IgM forming cells in the spleen gradually decreased up to day 21, the levels of P6-specific IgM in the plasma remained unchanged, up to day 21. Although the number of P6-specific IgG forming cells was still low, an elevated

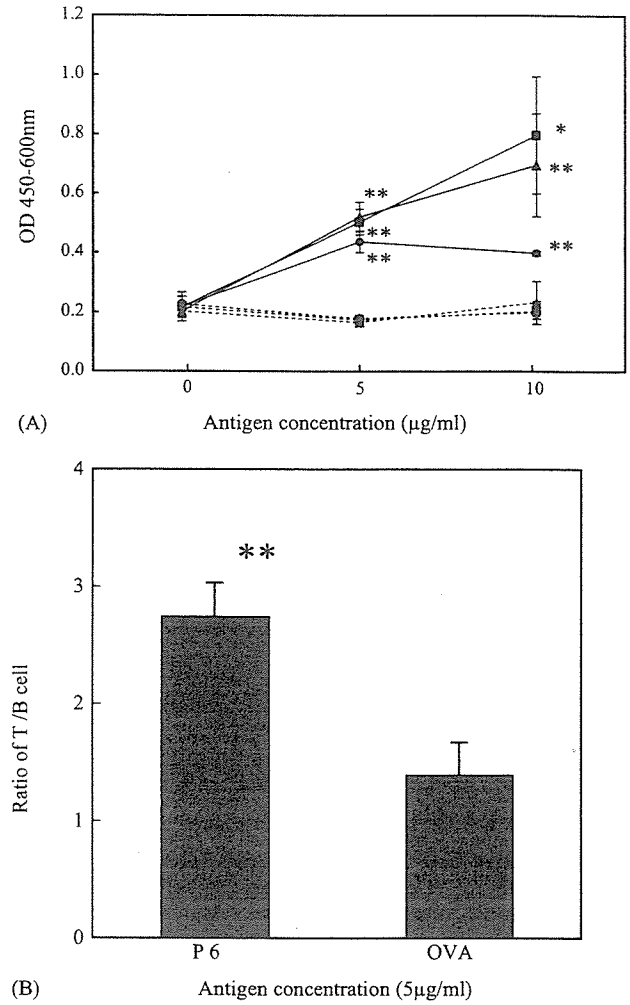

Fig. 2. In vitro cell proliferations using pulmonary lymph node (LN) cells from mice that received a single (closed circle), serial two (closed square) and serial three (closed triangle) IT challenge of OMP of nontypeable *Heamophilus influenzae* (NTHi) in the presence of P6 (solid line) or ovalbumin (OVA; broken line) were shown in Fig. 2A. LN cells were incubated with each antigen (0–10 μg/ml). Cells from pulmonary LNs were prepared from mice that received IT challenges of OMP as described in Section 2. $^*P<0.05$, $^{**}P<0.01$ (versus in the presence of OVA). The ratios of T/B cells in proliferated pulmonary LN cells are shown in Fig. 2B. The cultured pulmonary LN cells in the presence of P6 or OVA were stained with FITC-conjugated anti-mouse CD3 or PE-conjugated anti-mouse B220 mAb. $^{**}P<0.01$ (versus in the presence of OVA). Values represent mean ± S.D. of four animals.

level of P6-specific IgG in the plasma was noted at day 7. The number of P6-specific IgG forming cells in the spleen rapidly increased up to day 21 and the levels of P6-specific IgG peaked at day 21. Although an emergence of P6-specific IgG was noted at day 7, no detectable P6-specific IgG was found in BAL fluids at this time point. A slight increase in P6-specific IgG was, however, found in BAL fluids at days 14 and 21 (Fig. 4B). Although no increase in the number of P6-specific IgA forming cells was found in the spleen up to day 21, an increase in P6-specific IgA levels in BAL fluid was observed at day 21.

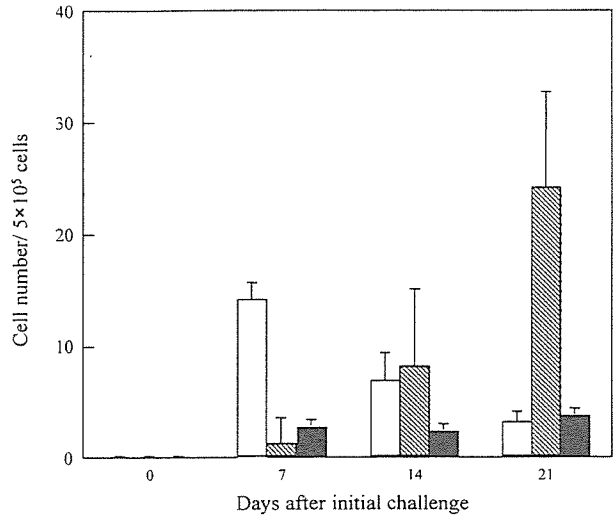

Fig. 3. Induction of P6-specific Ig M (open bar), IgG (slash bar) and IgA (closed bar) antibody-forming cells (AFCs) in spleen from mice before and after repeated IT challenges of outer membrane protein (OMP) three times with a 1-week interval. Spleen cells were prepared from mice at 7, 14 and 21 days after the initial challenge of OMP. Values represent mean no. of P6-specific AFCs ± S.D. of five animals.

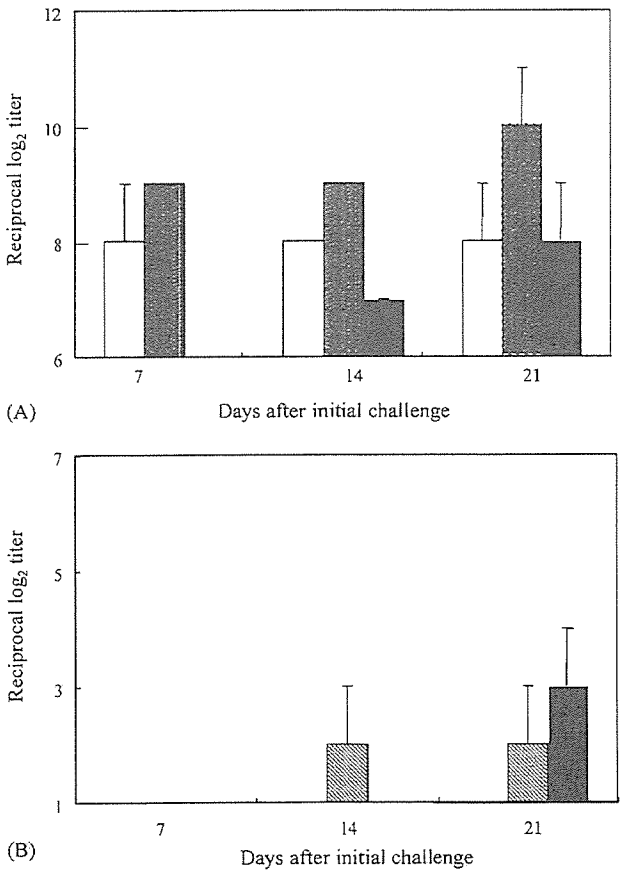

Fig. 4. Induction of P6-specific IgM (open bar), IgG (slash bar) and IgA (closed bar) in plasma (A) and bronchoalveolar lavage (BAL) fluid (B) in mice after repeated IT challenges by outer membrane protein (OMP) three times with a 1-week interval. Plasma and BAL fluid were obtained from mice at 7, 14 and 21 days after the initial challenge of OMP. Values represent mean antibody titer ± S.D. of five animals.

3.4. Histopathology of lung tissue

While neither inflammatory infiltration nor an accumulation of lymphoid cells was found in the lung parenchymea of untreated mice (Fig. 5A), a marked accumulation of mononuclear cells was observed in the peribronchial and periarterial tissue of mice that had received repeated IT challenges of OMP at day 21 (Fig. 5B). On the other hand, IgA was only detected on the surface of the bronchial epithelium of untreated mice (Fig. 5C). The number of cells that were positive for IgA in the subepithelial tissue gradually increased through days 14–21. Mononuclear cells beneath the bronchial epithelium in lung tissue of mice that had received repeated IT challenges of OMP stained positive for IgA at day 14 (Fig. 5D). The number of these IgA producing cells beneath the bronchial epithelium were increased at day 21 (Fig. 5E).

3.5. Bacterial clearance

We next attempted to determine whether repeated IT challenges of OMP led to an enhanced bacterial clearance of the NTHi strain in the lung tissue. A minimal, but significant decrease in bacterial density in lung tissue was found 1 h after bacterial challenge in mice that had received repeated IT challenges of OMP, compared with mice that received an IT challenge of PBS alone or in untreated mice ($P < 0.01$, Fig. 6). Approximately one log reduction in bacterial densities in the lung tissue was found 4 h after the bacterial challenge in OMP-treated mice compared with PBS-treated mice or untreated mice ($P < 0.01$). While bacterial densities in the lung tissue declined to around 10^6 cfu/g in PBS-treated mice or untreated mice 8 h after the bacterial challenge, no live bacteria were detected in the lung tissue of OMP-treated mice at the same time point.

4. Discussion

The findings herein demonstrate that repeated IT challenges lead to a decrease in the migration of mature DC into the pulmonary LN, although a rapid migration of mature DC into the pulmonary LN was found immediately after the initial IT challenge of OMP (Fig. 1A). The reduced migration of mature DC as the result of repeated IT challenges of OMP suggests that DC migration into the pulmonary LN is physiologically regulated. Our preliminary data indicate that the expression of CCL19 or CCL21 mRNA in pulmonary LN cells before and after IT challenge of OMP is unaltered, and a significant decrease in CCR7 mRNA expression in the pulmonary LN occurs 7 days after the initial IT challenge (data not shown). Since CCR7 is expressed by the naïve T cells as well as mature DC, the decreased expression of CCR7 mRNA in the pulmonary LN is not solely responsible for the attenuated migration of matured DC into the pulmonary LN. The precise mechanism for the regulation

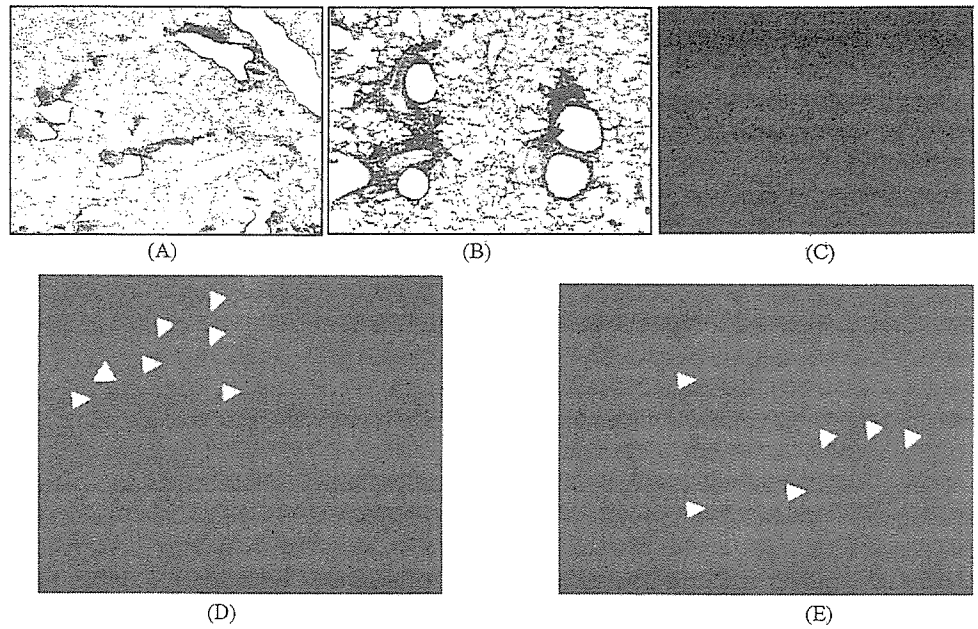

Fig. 5. Histological changes in lung tissue stained with hematoxylin–eosin and the localization of IgA in the lung tissue by an immunofluorescent method before and after IT challenges by outer membrane protein (OMP) three times at 1-week intervals. No mononuclear cell infiltration was found in the lung tissue of untreated mice (A), and a marked mononuclear cell infiltration was observed in peribronchial and periarterial tissues (arrows: B) (original magnification: ×40). IgA was detected only on the surface of bronchial epithelium of untreated mice (C). A gradual increase in the number of IgA positive cells (arrow heads) was found in mice that received repeated IT challenge through day 14 (D) to day 21 (E) (original magnification: ×200).

of DC migration into the pulmonary LN, therefore, remains unclear.

The appearance of mature DC in the pulmonary LN after the initial IT challenge of OMP appears to be correlated with in vitro P6-specific T cell proliferation. Although the absolute numbers of mature DC declined after repeated IT challenges of OMP, an in vitro P6-specific T cell proliferation was enhanced after the repeated IT challenge. These data indicate that an additional influx of mature DC is not required for the enhancement in P6-specific T cell proliferation in the pulmonary LN, or for the subsequent induction of P6-specific antibodies in the airways as well as systemic circulation after the repeated IT challenge. The induction of P6-specific IgG and M in plasma can be attributed to the appearance of P6-specific IgG and IgM forming cells in the spleen after a single or the repeated IT challenge of OMP. On the other hand, the increased production of P6-specific IgA found in BAL fluid at day 21 can be explained by a gradual increase in IgA producing cells in the subepithelial tissue shown at days 14 and 21.

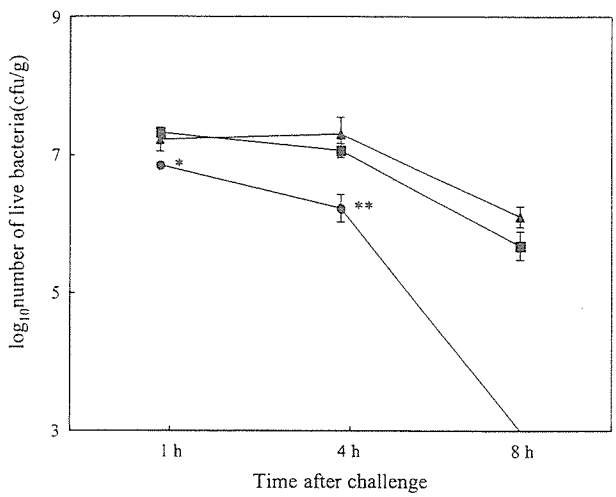

Fig. 6. The effect of repeated intratracheal (IT) challenges by outer membrane protein (OMP) three times at 1-week intervals on the bacterial clearance of nontypeable *H. influenzae* (NTHi) from the lung. NTHi strain at a dose of 1×10^8 cfu/head was intranasally challenged to mice that had previously received an intranasal challenge of NTHi OMP (closed circle) or sterile PBS (closed square), three times at 1-week intervals, or untreated mice (closed triangle). Quantitative bacterial cultures of lung tissue in infected mice were performed 1, 4 and 8 h after intranasal challenge with a live NTHi strain. Values represent the mean $\log_{10}$ cfu/g ± S.D. for five animals. $^{*}P < 0.05$, $^{**}P < 0.01$ (versus the untreated or PBS-treated mice).

Several previously published studies have reported that P6-specific antibodies raised in immunized mice are functional. Two investigators reported that the P6-specific antibodies were bactericidal and protective in a murine model of otitis media [9,11]. Another group reported that intranasal immunization with P6 and cholera toxin enhanced the nasopharyngeal clearance of NTHi in mice [12]. The above authors demonstrated that nasopharyngeal washings from immunized mice inhibited bacteria from adhering to cultured human epithelial cells, although no correlation was found between the levels of P6-specific IgA in nasal washings and bacterial clearance from the nasopharynx of mice. Bacterial clearance in the lung was significantly enhanced in mice that had received repeated IT challenges of OMP in this study. An interesting finding is a slight, but significant reduction

in bacterial density in the lung one hour after a challenge of NTHi in OMP-treated mice, compared to untreated or PBS-treated mice. The enhanced bacterial clearance can not be explained by the influx of neutrophils into the airway because several hours are required to observe neutrophil migration in the BAL fluids of mice that receive an IT challenge of gram-negative bacteria [21,25]. P6-specific IgA in BAL fluids, therefore, might inhibit the attachment of homologous strains on the airway epithelium of OMP-treated mice [26,27]. A recent study demonstrated that the proliferative response of peripheral lymphocytes to P6, in patients with COPD, was associated with a relative protection from exacerbation due to NTHi [28]. This report supports the findings herein, of a strong linkage between P6-specific cell proliferation in the pulmonary LN and the enhanced bacterial clearance of NTHi from the lung, which is associated with P6-specific immune response in mice after repeated IT challenges of OMP.

In conclusion, although the migration of mature DC into the pulmonary LN declined, P6-specific antigen presentation by DC in the pulmonary LN was enhanced after repeated IT challenges of OMP. P6-specific IgA in the airways as well as P6-specific IgG in the systemic circulation was subsequently induced in these mice, and was associated with an improved bacterial clearance of the homologous NTHi strain. Further studies will be required to clarify the mechanism(s) by which DC migration into the pulmonary LN declines after repeated IT challenges of OMP.

Acknowledgements

We are grateful to N. Kitajima, M. Magome and M. Fukahori for their skillful assistance, and to K. Fujihashi (University of Alabama at Birmingham, AL, USA) for his instructions concerning the ELISPOT assay. This study was supported by a Grant-in-aid for Scientific Research (C: 14570556) from the Ministry of Education, Science and Culture, Japan.

References

[1] Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of the art review. Clin Microbiol Rev 2001;14:336–63.
[2] Murphy TF. Bacterial otitis media: pathogenic considerations. Pediatric Infect Dis J 2000;19:S9–S16.
[3] Ubukata. Problems associated with high prevalence of multidrug-resistant bacteria in patients with community-acquired infections. J Infect Chemother 2003;9:285–91.
[4] Mylnarczyk G, Mlynarczyk A, Jeljaszewicz J. Epidemiological aspects of antibiotic resistance in respiratory pathogens. Int J Antimicrobial Agents 2001;18:497–502.
[5] Poolman JT, Bakaletz L, Cripps A, Danoel PA, Forsgren A, Kyd J, et al. Developing a nontypeable *Heamophilus influenzae* (NTHi) vaccine. Vaccine 2001;19:S108–15.
[6] Murphy TF, Nelson MB, Dudas KC, Mylotte J, Apicella MA. Identification of a specific epitope of *Haemophilus influenzae* on a 16,600-dalton outer membrane protein. J Infect Dis 1985;152:1300–7.
[7] Murphy TF, Bartos LC, Campagnari AA, Nelson MB, Apicella MA. Antigenic characterization of the P6 protein of nontypeable *Haemophilus influenzae*. Infect Immun 1986;54:774–9.
[8] Bertot GM, Becker PD, Guzman CA, Grinstein S. Intranasal vaccination with recombinant P6 protein and adamantylamide dipeptide as mucosal adjuvant confers efficient protection against otitis media and lung infection by nontypeable *Heamophilus influenzae*. J Infect Dis 2004;189:1304–12.
[9] Sabirov A, Kodama S, Hirano T, Suzuki M, Mogi G. Intranasal immunization enhances clearance of nontypeable *Heamophilus influenzae* and reduces stimulation of tumor necrosis factor alpha production in the murine model of otitis media. Infect Immun 2001;69:2964–71.
[10] Kodama S, Suenaga S, Hirano T, Suzuki M, Mogi G. Induction of specific immunoglobulin A and Th2 immune responses to P6 outer membrane protein of nontypeable *Haemophilus influenzae* in middle ear mucosa by immunization. Infect Immun 2000;68:2294–300.
[11] Badr WH, Loghmanee D, Karalus RJ, Murphy TF, Thanavala Y. Immunization of mice with P6 of nontypeable *Haemophilus influenzae*: kinetics of the antibody response and IgG subclasses. Vaccine 2000;18:29–37.
[12] Hotomi M, Saito T, Yamanaka N. Specific mucosal immunity and enhanced nasopharyngeal clearance of nontypeable *Heamophilus influenzae* after intranasal immunization with outer membrane protein P6 and cholera toxin. Vaccine 1998;16:1950–6.
[13] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
[14] Reis e Sousa C, Sher A, Kaye P. The role of dendritic cells in the induction and regulation of immunity to microbial infection. Curr Opin Immunol 1999;11:392–9.
[15] Cella M, Engering A, Pinet V, Pietes J, Lanzavecchia A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature 1997;388:782–7.
[16] Itakura M, Tokuda A, Kimura H, Nagai S, Yoneyama H, Onai N, et al. Blockade of secondary lymphoid tissue chemokine exacerbates *Propionibacterium acnes*-induced acute lung inflammation. J Immunol 2001;166:2071–9.
[17] McWilliam AS, Nelson D, Thomas JA, Holt PG. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. J Exp Med 1994;179:1331–6.
[18] Chan VWF, Kothakota S, Rochan MC, Panganiban-Lustan L, Gradner JP, Wachowicz MS, et al. Secondary lymphoid-tissue chemokine (SLC) is chemotactic for mature dendritic cells. Blood 1999;93:3610–6.
[19] Yanagihara S, Komura E, Nagafune J, Watarai H, Yanagihara Y. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. J Immunol 1998:3096–102.
[20] Yoshida R, Nagira M, Imai T, Baba M, Takagi S, Tabira Y, et al. EBL1-ligand chemokine (ELC) attracts a broad spectrum of lymphocytes: activated T cell strongly up-regulate CCR7 and efficiently migrate toward ELC. Int Immunol 1998;10:901–10.
[21] Satoh S, Oishi K, Iwagaki A, Senba M, Akaike A, Akiyama M, et al. Dexamethasone impairs pulmonary defense against *Pseudomonas aeruginosa* through suppressing iNOS gene expression and peroxynitrite production in mice. Clin Exp Immunol 2001;126:266–73.
[22] Jhonson KG, McDonald IJ, Perry MB. Studies on the cellular and free lipopolisaccharides from *Branhamella catarrhalis*. Can J Microbiol 1976;22:460–7.
[23] Kurono Y, Yamamoto M, Fujihashi K, Kodama S, Suzuki M, Mogi G, et al. Nasal immunization induces *Haemophilus influenzae*-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. J Infect Dis 1999;180:122–32.
[24] Fujihashi K, McGhee JR, Beagley KW, McPherson DT, McPherson SA, Huang C-H, et al. Cytokine-specific ELISPOT assay. Single cell

analysis of IL-2, IL-4 and IL-6 producing cells. J Immunol Method 1993;160:181–9.
[25] Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Goodman RE, Standiford TJ. Neutralization of IL-10 increases survival in a murine model of *Klebsiella pneumonia*. J Immunol 1995;155:722–9.
[26] Taylor DC, Cripps WA, Clancy RL. Inhibition of adhesion oh *Haemophilus influenzae* to buccal cells by respiratory secretions. Immunol Cell Biol 1990;68:335–42.
[27] Kurono Y, Shimamura K, Shigemi H, Mogi G. Inhibition of bacterial adherence by nasopharyngeal secretions. Ann Otol Rhinol Laryngol 1991;100:455–8.
[28] Abe Y, Murphy TF, Sethi S, Faden HS, Dmochowski J, Harabuchi Y, et al. Lymphocyte proliferative response to P6 of *Heamophilus influenzae* is associated with relative protection from exacerbations of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2002;165:967–71.

Epidemiol. Infect., Page 1 of 4. © 2006 Cambridge University Press
doi:10.1017/S0950268806007023 Printed in the United Kingdom

SHORT REPORT

Antimicrobial susceptibility and genetic characteristics of *Haemophilus influenzae* isolated from patients with respiratory tract infections between 1987 and 2000, including β-lactamase-negative ampicillin-resistant strains

L. QIN[1], H. WATANABE[2]*, N. ASOH[2], K. WATANABE[2], K. OISHI[2], T. MIZOTA[1], AND T. NAGATAKE[2]

[1] *Department of Social Environment Medicine and* [2] *Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Japan*

(*Accepted 27 May 2006*)

SUMMARY

The minimum inhibitory concentration (MIC) of five antibiotics and the presence of resistance genes was determined in 163 *Haemophilus influenzae* isolates collected over 13 years (1987–2000) in four two-yearly sampling periods from patients with respiratory tract infections. The prevalence of β-lactamase-negative ampicillin-susceptible strains was approximately 80% over the sampling period although fewer strains (65·9%) were recovered in the period 1995–1997. TEM-1 type β-lactamase-producing strains were less frequent starting at 15·6% and declining to 2·2% in the final sampling period. Low-β-lactamase-negative ampicillin-resistant (BLNAR) strains were uncommon in 1987–1989 (2·2%), peaked to 19·5% in 1995–1997, but fell back to 11·1% by 2000. Fully BLNAR strains were not detected until the last sampling period (6·7%). The MICs of ampicillin, levofloxacin, cefditoren and ceftriaxone remained stable but there was an eight-fold increase in the MIC of cefdinir over the sampling period. Pulsed-field gel electrophoresis of DNA digests showed that three representative BLNAR strains were genetically distinct and 11 DNA profiles were identified among 17 low-BLNAR strains. These data suggest that the number of genetically altered BLNAR and low-BLNAR strains are increasing in Japan.

Haemophilus influenzae can cause a variety of infections, including otitis media, bronchitis, pneumonia and meningitis [1, 2]. In the past, the activity of two β-lactamases, TEM-1 and ROB-1, accounted for almost all isolates with decreased susceptibility to ampicillin [3]. At present, the global prevalence of β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* remains low [4, 5], but the proportion of clinical BLNAR isolates is rapidly increasing, and has now reached more than 20% in Japan [6]. The characteristics of antimicrobial resistance of these strains are a serious concern for clinical prescribing. BLNAR strains have a resistance mechanism that decreases the affinity of ampicillin for penicillin-binding proteins (PBPs) [7]. The resistance phenotypes are classified according to substitutions at three positions of the *ftsI* gene which mediates septal peptidoglycan sysnthesis allowing the classification of strains as BLNAR or low-BLNAR by PCR [8].

In total, 163 strains of *H. influenzae* were isolated from sputum from patients with respiratory tract infections in Nagasaki University and its affiliated hospitals. These strains were selected at random and were divided into four groups, 45 strains between 1987 and 1989, 32 between 1991 and 1993, 41 between 1995 and 1997, and 45 between 1998 and 2000. Strains were capsule typed by slide agglutination with antisera (Difco Laboratories, Detroit, MI, USA) and

* Author for correspondence: Dr H. Watanabe, Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
(Email: h-wata@net.nagasaki-u.ac.jp)

Table. *Annual changes of the prevalence of each resistance class and antimicrobial susceptibility to five antibiotics*

Year		BLNAS[a]	BLPAR[b]	Low-BLNAR[c]	BLNAR[d]		MIC_{80} of five antibiotics (μg/ml) AMP[e]	CFX[f]	CFR[g]	CFN[h]	LFX[i]
A	87–89	82·2	15·6	2·2	0	Total	0·5	0·008	0·25	0·016	0·032
						BLNAS	0·5	0·008	0·25	0·016	0·032
						BLPAR	16	<0·004	0·5	0·016	0·032
						Low BLPAR	1·0	0·008	0·5	0·016	0·032
B	91–93	78·1	6·3	15·6	0	Total	0·5	0·008	0·5	0·016	0·032
						BLNAS	0·5	0·008	0·25	0·016	0·032
						BLPAR	32·0	<0·004	0·25	0·008	0·032
						Low BLPAR	1·0	0·016	1·0	0·016	0·032
C	95–97	65·9	12·2	19·5	0	Total	0·5	0·008	1·0	0·016	0·032
						BLNAS	0·5	0·008	0·5	0·016	0·032
						BLPAR	32·0	<0·004	0·5	0·016	0·032
						Low BLPAR	1·0	0·016	1·0	0·032	0·032
D	98–00	80·0	2·2	11·1	6·7	Total	1·0	0·016	2·0	0·032	0·032
						BLNAS	0·5	0·016	1·0	0·032	0·032
						BLPAR	4·0	<0·004	0·13	0·008	0·063
						Low BLPAR	1·0	0·016	1·0	0·063	0·032
						BLNAR	1·0	0·125	8·0	0·125	0·032

[a] β-lactamase-negative ampicillin susceptible strains. [b] β-lactamase-producing ampicillin-resistant (TEM-1 type) strains. [c] Low β-lactamase-negative ampicillin-resistant strains. [d] β-lactamase-negative ampicillin-resistant strains. [e] Ampicillin. [f] Ceftriaxone. [g] Cefdinir. [h] Cefditoren. [i] Levofloxacin.

β-lactamase production was detected using a nitrocefin-impregnated disk (Becton Dickinson, Sparks, MD, USA). The minimum inhibitory concentration (MIC) of five antibiotics was determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [9]. The antibiotics were: ampicillin (Meiji Seika Kaisha, Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo), cefditoren (Meiji Seika Kaisha), cefdinir (Astellas Pharma Inc., Tokyo) and ceftriaxone (Chugai Pharmaceutical Co., Tokyo).

PCR was performed to identify resistance genes using a multiplex assay as described previously [8]. Four sets of primers were obtained from Wakunaga Pharmaceutical Co. (Hiroshima, Japan): P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae*; TEM-1 primers to amplify a part of the bla_{TEM-1} gene; PBP3-S primers to identify an Asn526→Lys amino-acid substitution in the *ftsI* gene; and PBP3-BLN primers to identify an Asn526→Lys and Ser385→Thr amino-acid substitution in the *ftsI* gene.

Pulsed-field gel electrophoresis (PFGE) was performed as described previously [10] using *Sma*I digestion (Takara Shuzo Co., Shiga, Japan) and electrophoresis in a CHEF Mapper PFGE system (Bio-Rad Life Science Group, Hercules, CA, USA) was carried out at 6 V/cm with switch times of 0·47 and 63 s, and a run-time of 20 h. After staining with ethidium bromide, the interpretation of PFGE patterns was based on the criteria described by Tenover *et al.* [11]. Briefly, PFGE patterns were classified into four groups: identical in profile = indistinguishable; 1–3 bands difference = closely related; 4–6 bands difference = possibly related; and >7 bands difference = different.

The Table shows that of the 45 strains isolated from 1987 to 1989, 37 (82·2%) were classified as β-lactamase-negative ampicillin-susceptible (BLNAS) strains, seven strains produced TEM-1-type β-lactamase and were ampicillin resistant (BLPAR) and one strain was classified as low-BLNAR by PCR. The proportion of BLNAS strains fell in the ensuing two sampling periods but recovered in the final time period to 80%. The frequency of BLPAR strains fluctuated from 15·6% in the initial period through 6·3% and 12·2% to 2·2% in the final sampling period. Similar variation was observed for low-BLNAR strains with just 2·2% of strains expressing this phenotype in the first period but rising to almost 20% in the third period before falling back to 11%. Three BLNAR strains (6·7%) were detected only in the fourth sampling period. The respective MIC_{80} values (μg/ml) for the four periods against the strain collection

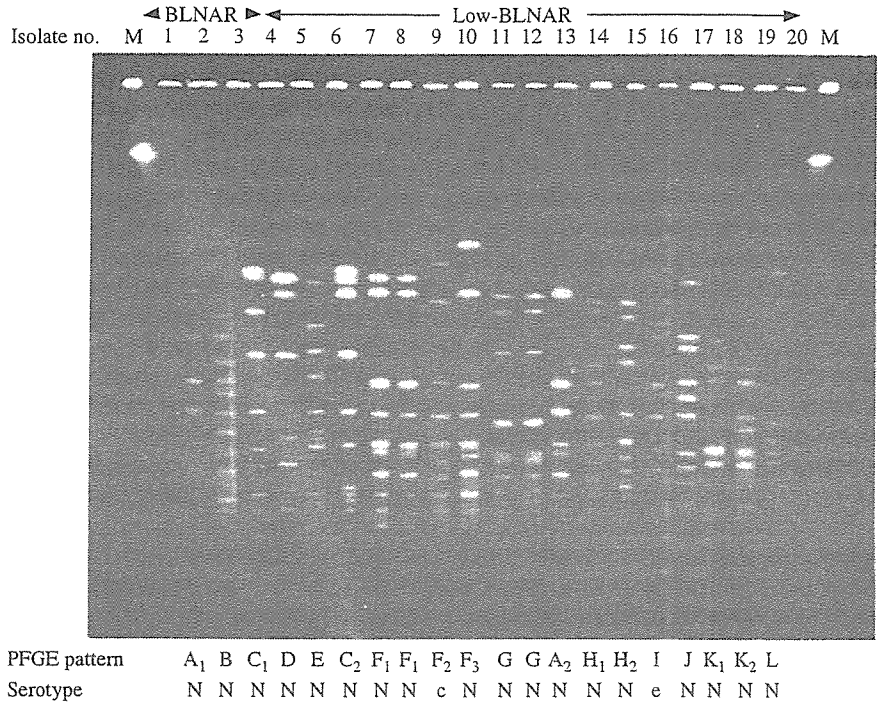

Fig. PFGE patterns of *Sma*I-digested DNA from three BLNAR and 17 low-BLNAR *H. influenzae* strains. Lanes 1–3, BLNAR strains; lanes 4–20, low-BLNAR strains. Coding of the PFGE patterns depicting group and subgroups and serotype status. N, Non-typable.

is also shown in the Table. The NCCLS susceptibility/resistant break-points for *H. influenzae* are 1 μg/ml for ampicillin, 2 μg/ml for levofloxacin, 1 μg/ml for cefdinir, and 2 μg/ml for ceftriaxone. The MIC of ampicillin, ceftriaxone, cefditoren and levofloxacin remained constant or within one doubling concentration over the years but resistance to cefdinir increased by eight-fold over the sampling period (Table).

The three BLNAR strains gave distinct DNA profiles in PFGE while 11 profiles were distinguished among the 17 low-BLNAR strains. Two of these profiles exhibited some similarities to profiles found in fully BLNAR strains. Three pairs of strains each exhibited similar patterns and four strains were grouped within the same pattern (Fig.). All but two of the 20 strains were non-typable with capsular antisera.

H. influenzae is one of the important pathogens associated with respiratory tract infections and thus acquisition of antimicrobial resistance raises concern. The prevalence of BLNAR strains was reported to be 2·4% in the United States between 2002 and 2003 [12], 1·3% in France in 1999 [13], and 9·3% in Spain between 1998 and 1999 [14]. Nevertheless, their global prevalence remains relatively low. However, BLNAR strains are spreading rapidly with increasing frequency in Japan with reported prevalence rates of 14·9% between 1996 and 1997 [15], and 23·1% between 1998 and 1999 [6], although BLNAR was identified by only MIC in these reports. We report here a prevalence of BLNAR strains of 6·7% by PCR between 1998 and 2000. An understanding of the characteristics of antimicrobial resistance of *H. influenzae*, especially BLNAR strains, is important not only for prescribing clinicians but also for formulation of practicable chemotherapy guidelines. Although *H. influenzae* strains are generally susceptible to the early cephalosporins [5, 12], the BLNAR and low-BLNAR strains recovered here showed a marginal increase in MIC to two of the three cephalosporins tested and an eight-fold increase in MIC of cefdinir which is consistent with a previous report from Japan [6].

PFGE of DNA macrorestriction fragments is a sensitive fingerprinting method for *H. influenzae* and this method was used by Karlowsky *et al.* [4] to demonstrate clonal dissemination of BLNAR strains in the United States between 2000 and 2001. However, the BLNAR and low-BLNAR strains found here displayed a variety of genetic backgrounds. It has previously been reported that *H. influenzae*, including resistant strains, can be transmitted at day-care centres or in the home [13, 16], and this may

be one reason for the spread of BLNAR strains in Japan. We did observe that some low-BLNAR strains isolated from different patients had similar PFGE patterns and therefore must consider that such strains could potentially spread in a community. Ongoing monitoring of *H. influenzae* resistance determinants is thought to be important and may help to predict how this organism responds to current antimicrobial regimens [17]. Despite the limitations of this pilot study, particularly the small sample size, it shows the value of surveillance of antimicrobial resistance levels and their genetic determinants in *H. influenzae* and further surveys specifically of BLNAR strains in the wider Japanese community should be undertaken to inform antimicrobial prescribing policy.

ACKNOWLEDGEMENTS

We thank Akihiro Wada (Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Japan), Chieko Shimauchi (Miyazaki Prefectural Nursing University, Japan) and Matsuhisa Inoue (Kitasato University School of Medicine, Japan) for their help in completing the PFGE studies. The study was supported by U.S.–Japan Cooperative Medical Science Program, Acute Respiratory Infections Panel.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Murphy TF, Apicella MA.** Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. *Reviews of Infectious Diseases* 1987; **9**: 1–15.
2. **Saito M, *et al.*** Clonal spread of an invasive strain of *Haemophilus influenzae* type b among nursery contacts accompanied by a high carriage rate of non-disease-associated strains. *Journal of Medical Microbiology* 2000; **49**: 845–847.
3. **Felmingham D, Washington J.** Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens – findings of the Alexander Project 1992–1996. *Journal of Chemotherapy* 1999; **11**: 5–21.
4. **Karlowsky JA, *et al.*** Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000–2001 leads to detection of clonal dissemination of a beta-lactamase-negative and ampicillin-resistant strain. *Journal of Clinical Microbiology* 2002; **40**: 1063–1066.
5. **Hoban D, Felmingham D.** The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. *Journal of Antimicrobial Chemotherapy* 2003; **50**: 49–59.
6. **Suzuki K, Nishimura T, Baba S.** Current status of bacterial resistance in the otolaryngology field: results from the Second Nationwide Survey in Japan. *Journal of Incfection and Chemotherapy* 2003; **9**: 46–52.
7. **Ubukata K, *et al.*** Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1693–1699.
8. **Hasegawa K, *et al.*** Rapidly increasing prevalence of β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. *Antimicrobial Agents and Chemotherapy* 2004; **48**: 1509–1514.
9. **National Committee for Clinical Laboratory Standards.** Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Wayne, PA: National Committee for Clinical Laboratory Standards, 1998.
10. **Yano H, *et al.*** Pulsed-field gel electrophoresis analysis of nasopharyngeal flora in children attending a day care center. *Journal of Clinical Microbiology* 2000; **38**: 625–629.
11. **Tenover FC, *et al.*** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* 1995; **33**: 2233–2239.
12. **Heilmann KP, *et al.*** Decreasing prevalence of beta-lactamase production among respiratory tract isolates of *Haemophilus influenzae* in the United States. *Antimicrobial Agents and Chemotherapy* 2005; **49**: 2561–2564.
13. **Dabernat H, *et al.*** *Haemophilus influenzae* carriage in children attending French day care centers: a molecular epidemiological study. *Journal of Clinical Microbiology* 2003; **41**: 1664–1672.
14. **Marco F, *et al.*** Antimicrobial susceptibilities of 1,730 *Haemophilus influenzae* respiratory tract isolates in Spain in 1998–1999. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 3226–3228.
15. **Seki H, *et al.*** Increasing prevalence of ampicillin-resistant, non-beta-lactamase-producing strains of *Haemophilus influenzae* in children in Japan. *Chemotherapy* 1999; **45**: 15–21.
16. **Watanabe H, *et al.*** Possible high rate of transmission of nontypeable *Haemophilus influenzae*, including β-lactamase-negative ampicillin-resistant strains, between children and their parents. *Journal of Clinical Microbiology* 2004; **42**: 362–365.
17. **Rennie RP, Ibrahim KH.** Antimicrobial resistance in *Haemophilus influenzae*: how can we prevent the inevitable? Commentary on antimicrobial resistance in *H. influenzae* based on data from the TARGETed surveillance program. *Clinical Infectious Diseases* 2005; **41** (Suppl. 4): S234–S238.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Vaccine 24 (2006) 826–834
Vaccine
www.elsevier.com/locate/vaccine

The phosphoprotein of attenuated measles AIK-C vaccine strain contributes to its temperature-sensitive phenotype

Katsuhiro Komase [a,*], Tetsuo Nakayama [b], Masumi Iijima [a], Kenji Miki [a,c], Ryuta Kawanishi [a,c], Hajime Uejima [b]

[a] *Division of Research and Development, Research Center for Biologicals, The Kitasato Institute, 6-111 Arai, Kitamoto-shi, Saitama 364-0026, Japan*
[b] *Laboratory of Viral Infection 1, Kitasato Institute for Life Science, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan*
[c] *Department of Neurology, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan*

Received 4 January 2005; received in revised form 11 June 2005; accepted 28 June 2005
Available online 15 August 2005

Abstract

Measles AIK-C vaccine strain exhibits temperature-sensitivity (*ts*). To identify the structural proteins, which contribute to the *ts* property of AIK-C virus, reverse genetics was used. MV-minigenome RNA was replicated at 32.5, 37, and 39 °C when the plasmids expressing N, P, and L proteins of the Edmonston strain (the parental strain of AIK-C) were used, whereas the minigenome RNA replicated only at 32.5 °C but did not at 37 °C and higher temperature when N, P, and L protein expression plasmids of the AIK-C strain were used. A series of minigenome experiments revealed that the amino acid substitution of leucine at position 439 of the P protein by proline (P439-Pro) contributes to the *ts* phenotype of AIK-C. Four recombinant viruses having various P genes were rescued from the modified AIK-C genome cDNA and *ts*-characteristics were compared in Vero cells by plaque formation assay. The results showed that the P439-Pro of AIK-C virus played a key role in the *ts* phenotype, but the other substitutions in the P gene might have an accessory function in the expression of the phenotype.

Keywords: Measles virus; Temperature-sensitivity; AIK-C vaccine strain

1. Introduction

Measles virus (MV) belongs to the order *Mononegavirales* with a nonsegmented negative-sense RNA genome having 15,894 nucleotides in length and encoding six structural proteins. The nucleoprotein (N), phosphoprotein (P), and large protein (L) associate with the RNA genome to shape the nucleocapsid structure. This ribonucleoprotein complex serves as a template for replication and transcription. Two external glycoproteins of the viral particle, hemagglutinin protein (H) and fusion protein (F), are responsible for viral attachment to the host cell and the fusion of viral and host cell membrane and after this step, the viral nucleocapsid penetrates into the cytoplasm. The matrix protein (M) is thought to contribute to the viral assembly and maturation. The P gene encodes two non-structural proteins; C and V proteins. The C protein is translated from the second AUG codon of the P mRNA [1]. The V protein is translated from V mRNA synthesized through a unique processing found in most *paramyxoviruses*, called RNA editing [2]. Recent studies showed that the C and V proteins were related to the viral replication and pathogenesis in vivo and played important roles to block interferon alfa/beta signal transduction [3–7].

Recently, two methods for direct genetic manipulation of the genome RNA of *Mononegavirales* have been established; the minigenome system and the infectious cDNA clone system. The former uses natural defective interfering (DI) genome RNA or artificial internally deleted genome RNA having reporter gene instead of virus component genes. Transcription and replication of minigenome RNA are driven by viral proteins, which are co-expressed by plasmids or

* Corresponding author. Tel.: +81 48 593 3953; fax: +81 48 593 3854.
E-mail address: komase-k@kitasato.or.jp (K. Komase).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.06.036

helper standard viruses. The latter system is based on a similar technology, but uses the authentic full-size genome cDNA so that the infectious recombinant viruses can be rescued [8–10]. These "reverse genetics" techniques are powerful tools not only for basic study on viral properties, such as viral protein characteristics, the mechanism of replication, transcription and pathogenesis, but also for practical purposes, such as the development of new vaccines and new viral vectors.

MV is a causative agent of measles as an acute illness in children and, in extremely rare incidence, slowly progressive and lethal disease of the central nervous system, known as subacute sclerosing panencephalitis (SSPE). After worldwide acceptance of efficient and safe live attenuated measles vaccines, number of measles cases drastically decreased in developed countries. Therefore, measles has been considered to be eradicable by vaccination [11]. Although the remarkable progress has been made by the Expand Programme on Immunization (EPI) organized by the World Health Organization (WHO), measles still ranks as one of the leading causes of the high mortality among children, especially under 9 months of age in developing countries [12].

The further attenuated live measles AIK-C vaccine strain was developed in 1974 from the Edmonston strain through passage in sheep kidney cells and chick embryo cells [13]. Since then, more than 15 million children have been immunized with AIK-C mainly in Japan and its efficacy and clinical safety have clearly shown [14]. Recently, the AIK-C strain has also been proven to be effective for children at 6 months of age or earlier. Accordingly, the AIK-C strain is one of the candidate vaccines for young infants at 6 months of age for high-risk groups in developing countries [15]. The AIK-C strain exhibits temperature-sensitivity (*ts*) [16]. Although little is known about how the virus becomes attenuated, it is widely accepted that *ts* phenotype related to viral attenuation mechanism. In this study, we identified the viral protein, which contributes to the *ts* phenotype using reverse genetics. Our data would benefit the further understanding of the nature of viral attenuation.

2. Materials and methods

2.1. Cells and viruses

HeLa and chick embryo fibroblast (CEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). Vero cells were grown in minimum essential medium (MEM) supplemented with 5% FCS. The Epstein–Barr virus-transformed marmoset B cell line B95a was cultured in RPMI1640 medium supplemented with 5% FCS. All cells were propagated at 37 °C in 5% CO_2. T7 RNA polymerase-expressing vaccinia virus vTF7-3 was kindly supplied by Dr. B. Moss (NIH, Bethesda, MD, USA) [17]. T7 RNA polymerase-expressing, replication-deficient vaccinia virus, MVAT7 pol, was a generous gift from Dr. G. Sutter (GSF Environment and Health Research Center, FRG) [18]. The AIK-C seed strain for vaccine production was used. AIK-C strain was propagated in CEF or Vero cells. The Edmonston strain was propagated in Vero cells.

2.2. cDNAs and sequence determination

The cDNA spanning the L gene of the AIK-C genome was synthesized by RT-PCR using specific primers, MVLF9239: 5′-gtccaagtggttccccgttatggac (mRNA sense) and MVLR15799: 5′-ccacctagggcaggattagggttcc (genome sense), referred by the results reported by Mori et al. [19]. The cDNA derived from the Edmonston strain (laboratory strain) was constructed by Schmid's method [20]. The 3′- and 5′-end sequences of the MV genome were amplified using the 3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA, USA) with MVSR340: 5′-tggagactccacaaataagg (genome sense) and MVSR635: 5′-cgtaaccgcctttgcgagcaag (genome sense) primers and the 5′-full RACE core set (Takara, Tokyo, Japan) with MVLF15752: 5′-gttagtcggatacagtgc (mRNA sense), MVLF15807: 5′-aatcctgccctaggtggtta (mRNA sense), MVLR15610: 5′-agtctagtatcagatagccgg (genome sense), and MVLR 15717: 5′-ggtctccttgactgttacctt (genome sense) primers, respectively. Nucleotide sequence was determined with primers at 300–350 base intervals along the AIK-C sequence using an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Plasmids

Three modified plasmids based on pUC19 were used for construction of the full-genome cDNA. Through a 4-step modification, *Xmn*I and *Nar*I sites of pUC19 were deleted and a *Not*I site was added just before the *Eco*RI site of the polylinker site. The resultant plasmid was designated pUC19M-5. *Ase*I and *Sca*I sites were deleted from pUC19M-5 to give pUC19M-7. The polylinker site of pUC19M-7 was exchanged with that of pNEB193 (NEB, Beverly, MA, USA) and the resultant plasmid, which carried *Not*I and *Pac*I sites on the polylinker site, was designated pUC193M-7. pCITE-K was constructed by deletion of the S.Tag, His.Tag sequences from pCITE 4a(+) plasmid (Novagen, Madison, WI, USA). pCITE-K plasmid has the T7 promoter, the Encephalomyocarditis virus internal ribosomal entry site (IRES), a multiple cloning site, a poly (A) signal and T7 terminator sequences. pCITE-K plasmid was used as a vector for construction of N, P, and L protein-expression plasmids.

2.4. Construction of MN, P and L protein-expression plasmids

The N, P and L cDNAs derived from the Edmonston and AIK-C strains were subcloned into pCITE-K plasmid, which was placed downstream of the T7 promoter and IRES sequence. The plasmids carrying the genes originating from

the Edmonston strain were designated pCIN01 (N gene), pCIP01 (P gene), and pCIL01 (L gene) and those bearing the genes derived from AIK-C, pCIAN01 (N gene), pCIAP01 (P gene) and pCIAL01 (L gene), respectively.

2.5. Construction of minigenome cDNA and synthesis of minigenome RNA

A cDNA template that could generate the negative-sense minigenome RNA was constructed. The cDNA, consisting of 1152 nts, contained the 3′-terminal 107 and 5′-terminal 109 nts corresponding to authentic MV genome RNA. These MV terminal sequences flanked a renilla luciferase open reading frame composed of 936 nt (Promega, Madison, WI, USA) [21]. The T7 RNA polymerase promoter and hepatitis delta virus ribozyme [22] are designed such that transcribed RNA created the specific 3′- and 5′-termini of the MV genome. Two T7 terminator sequences were aligned downstream from the ribozyme sequence to stop the RNA transcription efficiently by the T7 RNA polymerase [8]. The constructed cDNA was subcloned into pUC18 plasmid and the resultant plasmid was designated p18MGKLuc01. The negative-sense minigenome RNA was synthesized from the *Hin*dIII-digested p18MGKLuc01 plasmid by in vitro transcription using AmpliscribeTM T7 transcription kits (EPICENTRE TECHNOLOGIES, Madison, WI, USA) according to the supplier's instructions. The synthesized RNA was resuspended in RNase-free water.

2.6. Minigenome system

HeLa cells prepared in 12-well plates (about 1.5×10^5 cells/well) were infected with T7 RNA polymerase-expressing vaccinia virus, vTF7-3, at a multiplicity of infection (MOI) of 2–3. After 1 h adsorption, the cells were washed with Opti-MEM I (Invitrogen, Carlsbad, CA, USA) and then transfected with 0.5 μg of synthesized minigenome RNA and 0.5 μg of MV-N, 0.25 μg of MV-P and 0.1 μg of MV-L protein-expression plasmids using DMRIE-C reagent (Invitrogen, Carlsbad, CA, USA). After incubation at 32.5 °C for 3 h in 5% CO_2, the medium was replaced with fresh 5% FCS-DMEM containing cytosine arabinofuranoside (Ara-C) (40 μg/ml). After incubation at 32.5 or 37 or 39 °C for 40 h in 5% CO_2, the transfected cells were harvested and the assay for Renilla luciferase activity was done using a dual-luciferase assay system (Promega, Madison, WI, USA). The luciferase activity was quantitated by TopCount (Packard Instrument Company, Meriden, CT, USA).

2.7. Construction of a full-length cDNA genome corresponding to the AIK-C RNA genome

To construct a full-length cDNA genome corresponding to the complete 15,894 nucleotides of the AIK-C RNA genome, the 8 AIK-C cDNA fragments spanning the entire viral genome were combined. First, we added a *Not*I site/T7 promoter sequence just upstream of the leader sequence and a hepatitis delta virus ribozyme sequence immediately downstream of the trailer sequence by a PCR extension method using specific primers. Then the T7 terminator sequence/*Hin*dIII site was added behind the ribozyme sequence. These additional sequences were designed to generate the antigenomic RNA with the correct 3′- and 5′-termini of MV genome RNA under control of the T7 RNA polymerase. The 8 AIK-C cDNA fragments including a *Not*I/T7 promoter-leader and trailer-ribozyme-T7 terminator/*Hin*dIII sequences were ligated at appropriate restriction endonuclease sites. The plasmid having a full-length AIK-C cDNA genome was designated pMVAIK-C.

2.8. Rescue of the recombinant virus from cloned cDNA

B95a cells prepared in 12-well plates were infected with a vaccinia virus MVAT7 pol that expresses T7 RNA polymerase at an MOI of 10. MVAT7 pol was derived from a highly attenuated and host range restricted vaccinia virus, strain Ankara that is unable to propagate in human and most other mammalian cell lines [18]. After 1 h adsorption, the cells were washed with Opti-MEM and transfected with 0.5 μg of pCIAN01, 0.25 μg of pCIAP01, 0.1 μg of pCIAL01, and 1.5 μg of pMVAIK-C or derivatives of pMVAIK-C carrying various P genes using CellFectin transfection reagent (Invitrogen, Carlsbad, CA, USA). After incubation at 32.5 °C for 3 h, the medium containing transfection reagent/plasmid complex was replaced with fresh 5% FCS-DMEM. The tranfected cells were incubation at 32.5 °C in 5% CO_2 until cytopathic effect (CPE) could be observed in the transfected cells (about 5 days). The rescued viruses were passaged twice in B95a cells at 32.5 °C and the supernatants were harvested and stocked at −70 °C. The P genes of the four rescued viruses were amplified by the RT-PCR and were ascertained the nuclotide substitutions by direct sequence.

2.9. Evaluation of the ts phenotype of recombinant viruses

Replication of rescued recombinant viruses and the AIK-C vaccine strain and Edmonston strain were determined by plaque forming assay at 32.5, 36, 37, 38, 39 and 40 °C with Vero cells. Vero cells were prepared in 6-well plates and infected with serially diluted viruses. After incubation at permissive temperature (32.5 °C) for 1 h, the infected cells were washed with PBS twice, overlaid 2 ml of the 0.5% agarose contained 1% CS-MEM and then further incubated at various temperature indicated above for 6 days. Then, the cells were fixed with 2 ml of 0.5% Glutaraldehyde for 2 h. After removed the agar, the fixed cells were immunostained using anti-MV antibody to identify and enumerate the plaque by ABC staining using Vecta Stain elite ABC kit (Vector Laboratories, Burlingame, CA, USA).