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*i*NKT Cells Participate in the Exacerbation of Systemic Candidal Infection

Norihito Tarumoto, Yuki Kinjo, Naoki Kitano, Kazutoshi Shibuya, Shigefumi Maesaki, Yoshitsugu Miyazaki
[1]Department of Infectious Disease and Infection Control, Saitama Medical University
[2]Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases
[3]Department of Surgical Pathology, Toho University School of Medicine

Candida species are one major causal microorganism of hospital acquired bloodstream infections associated with high mortality. Phagocytes like neutrophils in innate immunity and CD4 T cells in acquired immunity have a major role in host defense immune response. It has been recently found that a type of innate-like lymphocyte called NKT cells respond against various organisms but its role in candidal infection remained unknown. Thus, we analyzed the role of NKT cells in the immune response against systemic candidiasis using mice deficient of NKT cells. *In vivo* studies revealed that invariant NKT cells play a limited role for controlling systemic candidal infection. On the other hand, studies looking at the role of glycolipid-activated NKT cells during candidal infection revealed that candida-infected mice injected with glycolipid had shorter survival period and greater number of fungal colonies in the kidney accompanied with reduced number of neutrophils in the blood and bone marrow. Surprisingly, glycolipid-mediated exacerbation of candidal infection was absent in IFN γ deficient mice. Co-infection of candida with intestinal commensals caused exacerbated infection in which IFN γ played a critical role in impairing fungal elimination. These results suggest that the excessive IFN γ released from candida and bacterial co-infection is a critical factor in worsening candidal infection.

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Case report

A case of pneumonia caused by *Legionella pneumophila* serogroup 12 and treated successfully with imipenem

Midori Nishizuka [a], Hiroki Suzuki [a,*], Tomoka Ara [a], Mari Watanabe [a], Mami Morita [a], Chisa Sato [a], Fumihiro Tsuchida [a], Junji Seto [b], Junko Amemura-Maekawa [c], Fumiaki Kura [c], Hiroaki Takeda [a]

[a] *Department of Respiratory Medicine, Saiseikai Yamagata Saisei Hospital, Japan*
[b] *Department of Microbiology, Yamagata Prefectural Institute of Public Health, Japan*
[c] *Department of Bacteriology I, National Institute of Infectious Diseases, Japan*

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ABSTRACT

The patient was an 83-year-old man hospitalized for *Haemophilus influenzae* pneumonia, who developed recurrent pneumonia after improvement of the initial episode. *Legionella pneumophila* serogroup 12 was isolated from the sputum, accompanied by increased serum antibody titers to *L. pneumophila* serogroup 12. Therefore, the patient was diagnosed as having *Legionella* pneumonia caused by *L. pneumophila* serogroup 12.

Case reports of pneumonia caused by *L. pneumophila* serogroup 12 are rare, and the case described herein is the first report of clinical isolation of this organism in Japan. When the genotype was determined by the protocol of The European Working Group for *Legionella* Infections (Sequence-Based Typing [SBT] for epidemiological typing of *L. pneumophila*, Version 3.1), the sequence type was ST68. Imipenem/cilastatin therapy was found to be effective for the treatment of *Legionella* pneumonia in this patient.

1. Introduction

Legionella infection is caused by organisms of the genus *Legionella*, which are Gram-negative bacilli. *Legionella pneumophila* strains are the predominantly isolated in clinical practice. *L. pneumophila* has been classified into 15 serogroups, of which serogroup 1 is most frequent cause of *Legionella* pneumonia, whereas *L. pneumophila* serogroup 12 is rarely responsible. Only four cases of pneumonia caused by *L. pneumophila* serogroup 12 have been reported to date [1–4]. Herein, we report a case of pneumonia caused by *L. pneumophila* serogroup 12, which is the first report of clinical isolation of this organism in Japan.

2. Case report

The patient was an 83-year-old man with a 3-day history of cough, sputum expectoration and anorexia, who was brought to our hospital by ambulance because of weakness of both legs in the beginning of January, 2012; he was then admitted to the hospital with the diagnosis of pneumonia. The patient had underlying diabetes mellitus, and had been on treatment with oral prednisolone 10 mg/day for the interstitial pneumonia. He gave no history of visits to hot spring facilities or circulation-type baths. Gram staining of the sputum on admission revealed phagocytosed Gram-negative bacilli, and sputum culture grew *Haemophilus influenzae*. The patient improved after 10 days of treatment with piperacillin/tazobactam (TAZ/PIPC) (12.5 g/day). Because the patient also had concomitant asthmatic symptoms, the steroid dose was increased to 80 mg of methylprednisolone/day (Fig. 1). Chest radiography carried out on the 13th hospital day revealed infiltrative opacities in the right upper lung field, with deterioration of the infiltrative opacities in the lower lung fields bilaterally that had improved once with TAZ/PIPC (Fig. 2A). Chest computed tomography (Fig. 2B and C) also confirmed the presence of infiltrative opacities in the right upper and both lower lobes. Anorexia was the only subjective symptom, and there was no diarrhea. The patient was recorded to have fever (body temperature, 38 °C) only on the 18th hospital day. The patient received oxygen supplementation at 2 L/min by nasal

* Corresponding author. Department of Respiratory Medicine, Saiseikai Yamagata Saisei Hospital, 79-1, Okimachi, Yamagata-city, Yamagata 990-8545, Japan. Tel.: +81 023 682 1111; fax: +81 023 682 0123.
E-mail address: hiroki-s@ma.catvy.ne.jp (H. Suzuki).

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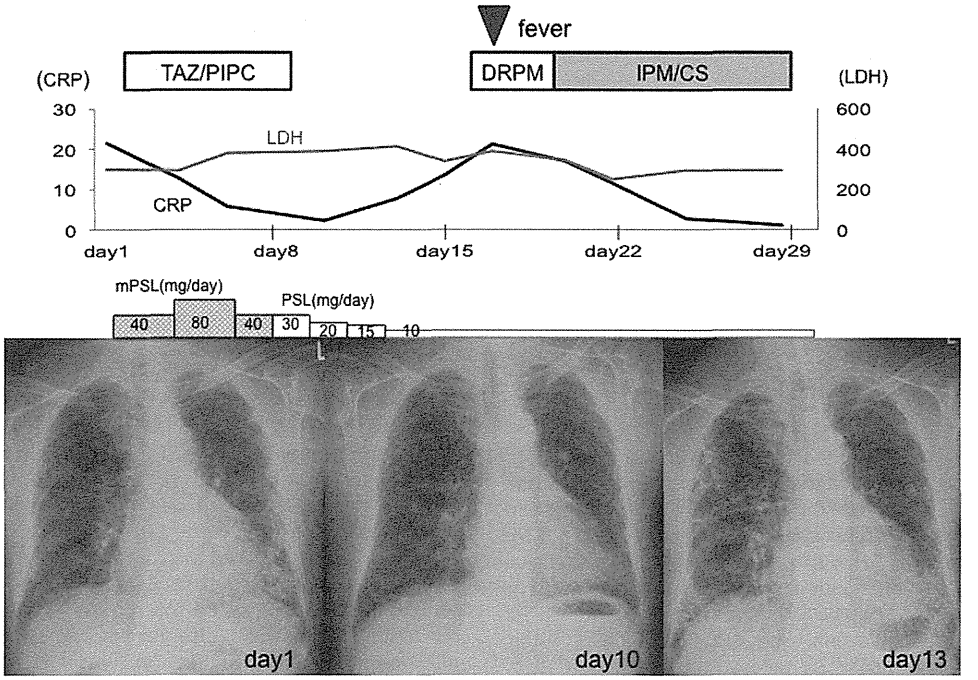

TAZ/PIPC piperacillin/tazobactam, *DRPM* doripenem, *IPM/CS* imipenem/cilastatin, *mPSL* methylprednisolone, *PSL* prednisolone

Fig. 1. Clinical course.

cannula; however, the oxygen flow needs to be increased temporarily to 3 L/min from 17th hospital day to 26th hospital day. Laboratory data obtained on the 13th hospital day were as follows: WBC count 17,400 cells/μL (stab cells 2.0%, segmented cells 89.0%), and serum CRP 7.63 mg/dL, revealing a tendency towards increase in the values of these parameters. Serum LDH was increased to 416 IU/L. The other parameters were BUN 24.0 mg/dL, serum creatinine 1.08 mg/dL, Na 136 mEq/L, K 3.8 mEq/L, Cl 98 mEq/L, fasting blood glucose 113 mg/dL, and HbA1c 6.2%. Gram staining of the sputum on the 13th hospital day showed phagocytosed Gram-negative bacilli. Doripenem (750 mg/day) therapy was initiated on the 17th hospital day. The

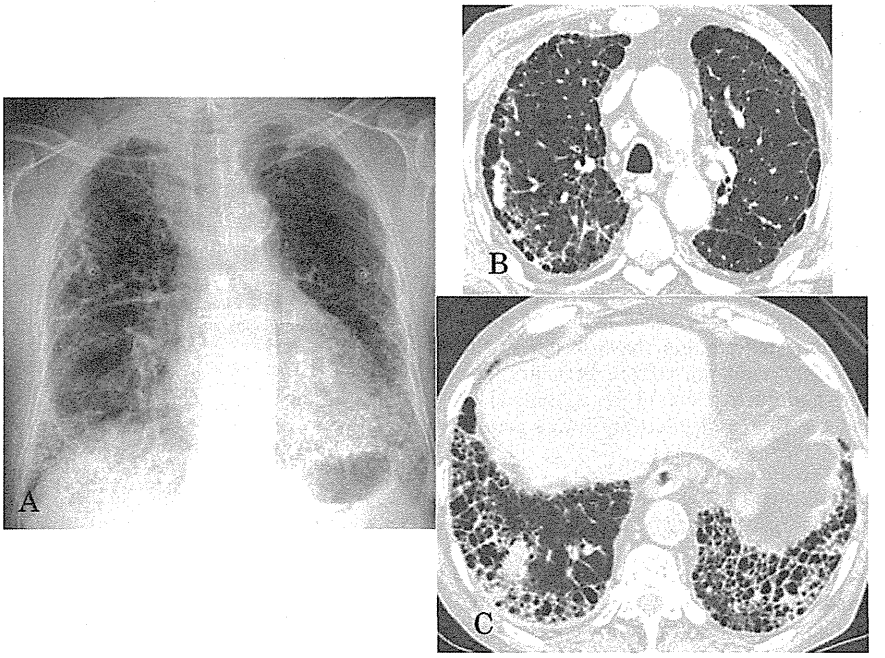

Fig. 2. Chest radiography and chest computed tomography on the 13th hospital day demonstrates the infiltrative opacities in the right upper and both lower lobes.

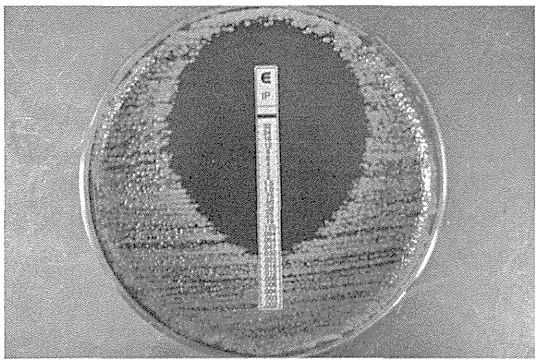

Fig. 3. Result of imipenem Etest for *Legionella pneumophila*.

doripenem therapy was switched to imipenem/cilastatin (1.5 g/day) therapy on the 20th hospital day because of elevation of the hepatic enzyme levels, and the patient's clinical condition improved thereafter. Because sputum obtained on the 13th hospital day cultured by the conventional method did not reveal any growth of bacteria, BCYE agar culture was carried out, which grew *Legionella* colonies on the 32nd hospital day. The MIC of imipenem for the isolated strain using Etest (AB Biodisk, Solna, Sweden) was 0.047 μg/ml when measured by the method of Murakami et al. [5] (Fig. 3). The slide agglutination test carried out using monovalent immune sera (Denka Seiken, Tokyo, Japan) identified the *Legionella* bacterium isolated from the sputum as *L. pneumophila* serogroup 12. The serum antibody titer on the 32nd hospital day was determined using microplate agglutination test kit (Denka Seiken, Tokyo, Japan) and in-house heat-killed *L. pneumophila* antigens (serogroups 7–15) [6]. The serum antibody titer determined was 1:512 for *L. pneumophila* serogroup 6 and 1:8192 for *L. pneumophila* serogroup 12, showing a distinct single high-titer. The serum antibody titers against *L. pneumophila* serogroups 1 to 5 and 7 to 11 and 13 to 15 were <1:16. A definitive diagnosis of infection caused by *L. pneumophila* serogroup 12 was made in our patient because *L. pneumophila* serogroup 12 was isolated from the sputum, accompanied by an increase of the serum antibody titer to *L. pneumophila* serogroup 12. Urinary *Legionella* antigen (BinaxNOW®) was negative throughout the course of the illness. The genotype was determined according to the protocol of the European Working Group for *Legionella* Infections (EWGLI; http://www.ewgli.org/) (Sequence-Based Typing (SBT) for epidemiological typing of *L. pneumophila*, Version 3.1). The results showed (3, 13, 1, 28, 14, 9, 3) for (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*), and the sequence type was ST68.

Because the possibility of nosocomial infection was considered to be highly likely in the patient, testing of swabs obtained from 13 sites in the hospital, including faucets and showerheads in the bathrooms of the ward, and environmental culture tests, including of the shower water, were performed. None of the cultures grew *Legionella* organisms, and the source of infection in this patient remained unclear.

3. Discussion

Fifty-seven species of *Legionella* are currently known (http://www.bacterio.net/legionella.html, as of January 22, 2014), among which *L. pneumophila* is the most frequently encountered causative organism of *Legionella* pneumonia. Among the 15 serogroups of *L. pneumophila*, serogroup 12 was first identified in 1987 [1]. Among the 5370 clinical strains whose genotypes are registered in the EWGLI database, there are 11 strains of serogroup 12 including our case as of January 21, 2014. Our present case is the first report of clinical isolation of this serogroup in Japan. Recently, 2 new cases of infection with *L. pneumophila* serogroup 12 including our case were recorded in the surveillance data of legionellosis in Japan [7]. A review of the literature collected within the scope of our search yielded 4 cases of pneumonia caused by serogroup 12 [1–4]. Clinical data were available for 2 of these patients, both of whom were cases of nosocomial infection occurring in immunosuppressed patients, just as in our patient [1,2].

The SBT proposed by the EWGLI is a technique to determine the base sequence by PCR amplification of some parts of 7 genes, i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*. The genotype of *L. pneumophila* in this case was ST68, which was found in 18 clinical strains in the EGWLI database as of January 21, 2014. Of these 18 strains, serogroup 6 accounted for the majority (11 strains), followed by serogroup 12 (4 strains). ST68 was previously isolated in 1 case of serogroup 6 in Japan, and therefore our case represents the second case of isolation of the ST68 genotype.

Our patient showed an increase of the serum antibody titer to *L. pneumophila* serogroup 6. Because cross-reactions between serogroups 6 and 12 have been reported previously [1], a cross-reaction in our patient also may occur. However, the case of infection with more than one *L. pneumophila* serogroup cannot be ruled out.

In a study by Murakami et al., the MIC of imipenem was 0.023–0.064 μg/ml for all 23 clinical isolates of *Legionella*, indicating good sensitivity of this organism to imipenem. The MIC for the isolate in our patient reported here was also within the above range. In general, it is considered that imipenem exerts no effect on *Legionella* bacteria *in vivo* because of its poor transfer into macrophages [8,9]. On the other hand, the efficacy of this drug for this infection has also been occasionally reported [10–15].

Ramirez JA et al. reported imipenem showed superior bactericidal activity against intracellular *L. pneumophila* compared to erythromycin in an *in vitro* model [13].

Our patient with *Legionella* pneumonia reported here improved with imipenem/cilastatin therapy, imipenem/cilastatin may be effective for the treatment of *Legionella* pneumonia in some cases.

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Original article

Contradiction between *in vitro* and clinical outcome: Intravenous followed by oral azithromycin therapy demonstrated clinical efficacy in macrolide-resistant pneumococcal pneumonia

Shigeru Kohno [a], Kazuhiro Tateda [b], Jun-ichi Kadota [c], Jiro Fujita [d], Yoshihito Niki [e], Akira Watanabe [f], Masahito Nagashima [g,*]

[a] *Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan*
[b] *Department of Microbiology and Infectious Diseases, Toho University Faculty of Medicine, Tokyo, Japan*
[c] *Department of Internal Medicine 2, Oita University Faculty of Medicine, Oita, Japan*
[d] *Department of Infectious, Respiratory, and Digestive Medicine, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan*
[e] *Department of Clinical Infectious Diseases, School of Medicine, Showa University, Tokyo, Japan*
[f] *Research Division for Development of Anti-Infective Agents, Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan*
[g] *Clinical Research, Development Japan, Pfizer Japan Inc., 3-22-7, Yoyogi, Shibuya-ku, Tokyo 151-8589, Japan*

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ABSTRACT

We conducted a multicenter, unblinded, non-comparative, phase 3 trial of azithromycin-intravenous therapy followed by oral administration in Japanese adults to evaluate clinical efficacy and safety against community-acquired pneumonia in order to obtain regulatory approval for the intravenous formulation in Japan. Azithromycin (500 mg, once daily) was intravenously administered for 2–5 days followed by oral 500 mg once daily administration to complete a total of 7–10 days treatment in 102 adults with moderate-to-severe community-acquired pneumonia. The efficacy rate in the Clinical Per Protocol Set overall was 84.5% (60/71 subjects) on Day 15 (primary analysis). The most common causative pathogen was *Haemophilus influenzae* (17 strains), followed by *Streptococcus pneumoniae* (14 strains), *Moraxella catarrhalis* (5 strains) and *Mycoplasma pneumoniae* (5 strains). Eleven of 14 *S. pneumoniae* isolates were resistant to azithromycin (MIC ≥2.0 μg/ml), of which 5 strains with a relatively low MIC of <32 μg/ml had only *mef* A gene and 6 strains with a high MIC of >64 μg/ml had only the *erm* B gene except for 2 isolates having both the *mef* A and *erm* B genes. Despite dominance of macrolide-resistant strains in Japan, clinical efficacy and bacterial eradication were achieved in 10 of 11 patients (90.9%). Intravenous-to-oral azithromycin therapy demonstrated excellent clinical and bacteriological effects on moderate-to-severe pneumococcal pneumonia despite a high MIC and resistance gene development. This discrepancy is referred to as the "*in vivo-in vitro* paradox". The current study results provide an insight into this paradox.

Registration number: NCT00809328

1. Introduction

Community-acquired pneumonia (CAP) is a common but serious lower respiratory tract infection and remains to be a major cause of morbidity and mortality despite a variety of therapeutic options available [1,2]. In Japan, pneumonia is the third leading cause of death with an incidence of 98.8 per 100,000 populations, which has shown an increasing trend [3]. In addition, the secondary bacterial pneumonia during an influenza epidemic is a major healthcare problem in patients with influenza infection, especially in the elderly and in those with co-morbidities such as obstructive pulmonary disease, cardiovascular disease, diabetes, and renal failure [4].

The most common causative pathogens for CAP are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and atypical pathogens including *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*, which are ranked among the top 4 pathogens both in Japan and the United States of America [2,4]. Initial antimicrobial therapy for CAP is usually empirical, and should provide wide-range coverage for major bacterial and atypical causative pathogens in

* Corresponding author. Tel.: +81 3 5309 7049; fax: +81 3 5309 9060.
E-mail address: masahito.nagashima@pfizer.com (M. Nagashima).

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CAP [2,4]. Furthermore, the initial empirical therapy is complicated by the increasing global prevalence of antibiotic resistance, in particular resistance to β-lactams, quinolones and/or macrolides, among the common causative pathogens in CAP.

The increasing rate of macrolide-resistant strains of *S. pneumoniae* with the macrolide efflux pump *mef* A genotype which gives rise to low-level macrolide resistance and/or the erythromycin ribosomal methylase *erm* B genotype which gives rise to high-level resistance leads to concern over the clinical efficacy of macrolides for the empirical treatment of CAP [5–8]. Recent study reported that in Japan, 81.4% (127/156) of *S. pneumoniae* isolates were resistant to erythromycin, and 50% had only the *erm* B gene, 23.7% had only the *mef* A gene, and 7.1% had both the *mef* A and *erm* B genes [6]. However, the clinical relevance of macrolide resistance has not been clearly established. A number of studies have reported the treatment failures with macrolides, breakthrough bacteremia, or both in patients infected with macrolide-resistant pneumococci [9–11]. In contrast, other studies have shown an apparent effectiveness of these agents in the treatment of CAP caused by macrolide-resistant *S. pneumoniae*, which suggests discordance between *in vitro* susceptibility and clinical efficacy of *in vivo* macrolide therapy [12,13]. This discrepancy has been referred to as the "*in vivo-in vitro* paradox" [14]. An open-label, non-comparative study in Japanese patients with mild to moderate CAP demonstrated the clinical effectiveness of a 3-day course of oral AZM formulation in the treatment of CAP with macrolide-resistant *S. pneumoniae* [15].

Azithromycin (AZM) is a macrolide antibiotic, having an extended spectrum of antimicrobial activity against both bacterial and atypical causative pathogens in CAP. The Infectious Diseases Society of America/American Thoracic Society (IDSA/ATS) consensus guidelines on the management of CAP in adults recommend AZM as the first-line treatment for outpatients without co-morbidity and AZM plus a β-lactam as the first choice for CAP patients requiring hospitalization (but not requiring intensive care unit [ICU]) treatment [4]. The guidelines also recommend a β-lactam plus either AZM or fluoroquinolone for hospitalized patients with severe CAP requiring ICU treatment. In patients with moderate or severe CAP, injectable antimicrobial agents are to be preferred when oral administration of antimicrobials is difficult due to gastrointestinal dysfunction, high fever, dyspnea, consciousness disturbance, and application of oxygen inhalation and ventilator. Switching from intravenous to oral therapy as soon as patients are clinically stable can reduce the length of hospitalization and lower the associated expenses.

We conducted a multicenter, unblinded, non-comparative phase 3 trial of intravenous therapy followed by oral AZM administration in Japanese adults to evaluate clinical efficacy and safety in the treatment of moderate to severe CAP requiring initial intravenous antibiotic therapy, which provides an insight into the "*in vivo-in vitro* paradox" against macrolide-resistant *S. pneumoniae*.

2. Materials and methods

This study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines, the principle of the Declaration of Helsinki and all applicable laws and regulations. The protocol was reviewed and approved by the Institutional Review Boards at all participating study sites. All subjects provided written informed consent before enrollment.

2.1. Study design

This multicenter, non-randomized, unblinded, non-comparative phase 3 study was designed to investigate the clinical efficacy and safety of AZM switch therapy, intravenous (IV) to oral formulation (AZM IV-to-oral switch therapy), in Japanese patients with moderate to severe CAP. An independent Data Review Committee (DRC) was organized to assure an objective and unified efficacy evaluation based on the clinical conditions and X-ray findings. All subjects received 500 mg IV AZM administrated once daily in infusate concentration of 1 mg/ml over 2 hours for 2–5 days, followed by 500 mg oral AZM (2 × 250 mg tablet) once daily to complete a total of 7–10 days. Switching from IV to oral therapy was determined by the investigators according to the subject status referring to the IDSA/ATS consensus guidelines (e.g., hemodynamical stability, improvement in clinical conditions, ability to ingest medications, and normally functioning gastrointestinal tract). The period of oral dosing was also determined at the discretion of the investigators according to the subject's conditions. The primary endpoint was clinical response assessed by the DRC at the end of treatment (EOT), and on Days 15 and 29. The primary analysis of the primary endpoints was to compute the efficacy rate from the clinical response on Day 15 in the Clinical Per Protocol Set (CPPS). The secondary endpoints were the following: the tendency toward clinical improvement at Day 3 assessed by the investigators; the bacteriological response on Day 3, at EOT, and on Days 15 and 29 assessed by the DRC; the bacteriological response assessed by the investigators; and the clinical response assessed by the investigators. However, the results of the latter 2 responses were not noted in this article as they showed a similar trend to the others.

2.2. Eligibility criteria

Subjects were enrolled in this study after written informed consent was obtained from the subject or a legally authorized representative. Men and women aged 16 years or older requiring initial IV antibacterial therapy were eligible if they were given a diagnosis of moderate to severe CAP that was confirmed by chest X-ray or Computed Tomography (CT) scan demonstrating a new pulmonary infiltrate. The following conditions were supportive for CAP diagnosis; existing inflammatory condition as increased white blood cell (WBC) counts and/or raised CRP, fever, cough, sputum, chest pain, rales and dyspnea. Severity of infection was evaluated according to the recommendation by the Japan Society of Chemotherapy [16]. The severity of pneumonia was evaluated on the basis of the standardized criteria such as body temperature, chest X-ray findings, peripheral WBC count, and CRP. Chest X-ray was the most important tool and therefore a precise interpretation of the finding was also necessary.

The exclusion criteria of the study included the following conditions or situations: known hypersensitivity or intolerance to AZM, or any macrolide or ketolide antibiotics, liver function impairment, severe renal impairment, a severe underlying disease or complication that precludes evaluation of therapy, treatment of systemic antimicrobials within 7 days prior to the initiation of the study medication (except if it was evaluated ineffective for CAP by the investigator), infection requiring concomitant use of systemic antimicrobial other than the study drug, pregnancy or lactation in women, immunocompromised state, primary lung cancer or lung metastasis from another cancer, or other conditions considered ineligible for the study by the investigators.

The following concomitant medications were prohibited until the primary evaluation period (Day 15): systemic antibiotics (oral, injection), human immunoglobulin, colony-stimulating factors (granulocyte-colony stimulating factor, macrophage-colony stimulating factor etc.), corticosteroid (systemic or inhalation 10 mg/day or over in prednisolone equivalent), taking analgesic antipyretic continuously (as-needed use is permissible, but more than 3 days

use is not permissible), other investigated drugs or medical devices, and bronchial lavage.

A study subject could be withdrawn from the study at any time for any of the following reasons: (1) The investigators considered that it was inappropriate to continue the study due to (i) insufficient clinical responses and/or biological responses; (ii) the occurrence of (serious) adverse events, and (iii) other reasons (including the cases where infections by causative pathogens that azithromycin was ineffective were confirmed); (2) it was found after the start of the study that the subject did not meet the inclusion criteria or met the exclusion criteria; and (3) the subject or the representative requested to withdraw from the study. If a subject withdrew from the study, and also withdrew consent for the disclosure of future information, no further evaluations were to be performed, and no additional data were to be collected. The sponsor could retain any data collected before such withdrawal of consent and continue to use them.

2.3. Clinical and radiological assessments

Clinical efficacy was assessed mainly based on the evaluation of chest X-ray (or CT) findings, body temperature, WBC count, CRP, signs and symptoms of CAP generally according to the criteria for the evaluation of effectiveness in clinical efficacy recommended by Japan Society of Chemotherapy [16]. The clinical response was generally evaluated as "effective" if improvements in 3 of the following 4 parameters of the criteria were achieved: (1) resolution of fever (body temperature $\leq$37°C); (2) normalization of WBC count (reduction to <9000 mm^3); (3) improvement in CRP (30% or more reduction from the previous levels); and (4) significant improvement in chest X-ray or CT findings. Chest X-ray was performed on Day 1 (prior to dosing), Day 3, at EOT, on Days 15 and 29, and chest CT scan was performed as needed.

The medical rationales for switching from IV to oral therapy were also investigated.

2.4. Bacteriological assessment

At the baseline visit, all subjects provided clinical specimens (sputum). The causative pathogens were determined by culture results, Geckler's classification of specimens, and observation of the bacteria inside phagocytic cells. All sputum samples were cultured except for subjects who did not have a sputum sample as prescribed in the protocol. Sputum samples were evaluated by Gram stain for purulence, defined as >25 WBCs and <10 squamous epithelial cells per field at 100× magnification. Bacterial cells were counted per field at 1000× magnification for a simple quantitative analysis. Purulent sputum, invasive respiratory, and blood samples were cultured at the local laboratories if needed. The clinical specimens were sent to a central laboratory for culture, and isolated pathogens were tested for susceptibility to AZM, erythromycin, ceftriaxone, sulbactam/ampicillin, tazobactam/piperacillin, minocycline, ciprofloxacin and levofloxacin (LVFX) according to the Clinical and Laboratory Standards Institute procedures. In addition, *S. pneumoniae* isolates were tested for susceptibility to penicillin G (PCG), and *H. influenzae* and *Moraxella catarrhalis* isolates for β-lactamase production. Genotyping was performed on AZM-resistant isolates of *S. pneumoniae* to determine the presence of *erm* gene and/or *mef* gene by PCR (for isolates with MIC >0.5 μg/ml). In addition, subjects were to submit a urine specimen for *S. pneumoniae* antigen testing (BINAX®) and *Legionella pneumophila* antigen testing (BINAX®). Most of the subjects underwent urinary antigen testing for both pathogens.

The specimens for detection of *M. pneumoniae*, and *C. pneumoniae* and *L. pneumophila* were sent to the central laboratory for testing. Laboratory workup for *M. pneumoniae* included sputum for PCR detection and culture and acute and convalescent serology by particle agglutination test. Laboratory workup for *C. pneumoniae* included oropharyngeal swab for PCR detection, culture, acute and convalescent serology by microimmunofluorescent-antibody assay. Laboratory workup for *L. pneumophila* included sputum for PCR detection, culture, acute and convalescent serology by indirect fluorescent antibody assay. Most of the subjects underwent acute and convalescent serology and PCR for *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*. Seroconversion was considered positive when a fourfold increase in antibody titer, in either immunoglobulin G and/or immunoglobulin M, was reported between the acute- and convalescent-phase serum specimens.

Causative pathogens in some subjects had not been identified, and these subjects received a diagnosis of "suspected bacterial pneumonia" or "suspected atypical pneumonia". The procedures for the diagnoses were based on 5 items for symptoms/findings and 1 item for pathological findings as follows: (1) under 60 years of age, (2) no or minor underlying diseases, (3) stubborn cough, (4) poor chest auscultatory findings, (5) no sputum, or no identified aetiological agent by rapid diagnosis, and (6) a peripheral WBC count below 10,000/μl [17].

Bacteriological response was assessed based on the criteria for the evaluation of effectiveness in bacteriological efficacy recommended by Japan Society of Chemotherapy [16]. Bacteriological response was assessed as "eradication" if the original pathogen was not identified in the sputum or other culturable specimen, "presumed eradication" if the subject was not producing sputum or other culturable specimen, "persistence" if the original pathogen remained in the sputum or other specimen, and "replacement bacterium" if the original pathogens were eradicated by treatment, other new pathogens appeared in the same specimen with symptoms and/or findings of an infection. The bacteriological response by each baseline causative pathogen was evaluated for multiple-pathogen bacterial pneumonia. After the evaluation by each baseline causative pathogen, the overall bacteriological response for each subject was evaluated by the DRC.

2.5. Safety assessment

All subjects who received at least 1 dose of the study drug were included in the safety analysis. Safety data were obtained from findings of clinical signs/symptoms, physical examinations, vital signs, and laboratory data up to 29 days. All adverse events, regardless of suspected causal relationship to the study drug, were recorded. The causality and severity of the adverse events were evaluated by the investigators based on MedDRA terminology.

2.6. Statistical analysis

A total of 100 subjects were targeted for treatment. The Per Protocol Set (PPS) was the primary analysis set for the efficacy analyses. In this study, the CPPS and the Bacteriologic Per Protocol Set (BPPS) were used as the PPS. The CPPS consisted of all subjects who received at least 1 dose of the study drug, had no significant violations of the protocol, and underwent the prescribed evaluations during the observation period as specified in the protocol. The BPPS consisted of all subjects in the CPPS in whom causative pathogens were identified by the culture, PCR and serology at baseline. The Full Analysis Set (FAS) consisted of all subjects who received at least 1 dose of the study drug.

For the primary analysis of the primary endpoints, the efficacy rate and its 95% confidence interval (CI) in the clinical response in the CPPS on Day 15 as evaluated by the DRC were calculated by

applying the Clopper–Pearson (Exact) method. For the secondary analysis of the clinical response evaluated by the DRC, the efficacy rate and 95% CI at EOT and on Day 29 in the CPPS and the efficacy rate and 95% CI on each evaluation day (EOT and Days 15 and 29) in the BPPS were also calculated. Safety analysis was performed for the "Safety Analysis Set," the population of subjects who received at least 1 dose of the study drug. Safety analysis was performed mainly by descriptive statistics.

3. Results

3.1. Subject disposition

Subjects were enrolled at 35 medical centers nationwide in Japan from February 2009 to March 2010. A total of 102 subjects were enrolled into the study, and all of them received the study drug. Of those receiving study medication, 29 subjects (28.4%) discontinued the study, and their breakdown was 18 subjects (17.6%) due to insufficient efficacy, 6 subjects (5.9%) due to adverse events, 3 subjects (2.9%) due to a deviation from the inclusion criteria, and 2 subjects (2.0%) due to voluntary withdrawal. The decision on discontinuation of the subjects was made by the principle investigator except for voluntary withdrawal. Baseline demographic characteristics by analysis set are shown in Table 1. The FAS consisted of 63 male subjects (61.8%) and 39 female subjects (38.2%), with male subjects accounting for the higher percentage. The mean age of the subjects was 55.4 years, with 43 subjects (42.2%) being 65 years or older, and 16 subjects (15.7%) being 75 years or older. Demographic characteristics did not differ markedly among the analysis sets.

Table 1
Baseline demographic characteristics by analysis set.

Characteristics	Analysis set		
	FAS	CPPS	BPPS
	(N = 102)	(N = 73)	(N = 33)
Age (year)			
16–44	29 (28.4)	23 (31.5)	15 (45.5)
45–64	30 (29.4)	21 (28.8)	6 (18.2)
65–74	27 (26.5)	20 (27.4)	10 (30.3)
75–79	13 (12.7)	7 (9.6)	2 (6.1)
80 or over	3 (2.9)	2 (2.7)	0
Mean ± SD	55.4 ± 18.6	54.0 ± 18.8	49.2 ± 19.6
Range	16–84	16–80	16–78
Gender			
Male	63 (61.8)	46 (63.0)	19 (57.6)
Female	39 (38.2)	27 (37.0)	14 (42.4)
Height (cm)			
Mean ± SD	161.7 ± 9.1	161.6 ± 9.1	162.2 ± 10.2
Range	140.0–184.0	140.0–184.0	140.0–184.0
Body Weight (kg)			
Mean ± SD	57.2 ± 10.8	57.0 ± 10.2	57.5 ± 10.3
Range	35.0–91.5	35.0–91.5	40.3–78.3
Body mass index[a] (kg/m^2)			
Mean ± SD	21.9 ± 3.8	21.8 ± 3.7	21.9 ± 3.6
Range	15.5–35.8	15.8–35.8	15.8–35.8
Current smoking status			
Never smoked	45 (44.1)	35 (47.9)	18 (54.5)
Ex-smoker	30 (29.4)	21 (28.8)	6 (18.2)
Smoker	27 (26.5)	17 (23.3)	9 (27.3)
Alcohol drinking			
Yes	43 (42.2)	28 (38.4)	14 (42.4)
No	59 (57.8)	45 (61.6)	19 (57.6)
Severity of CAP			
Mild	–[b]	8 (11.0)	4 (12.1)
Moderate	–[b]	64 (87.7)	29 (87.9)
Severe	–[b]	1 (1.4)	0

Abbreviations: FAS, full analysis set; CPPS, clinical per protocol set; BPPS, bacteriologic per protocol set, CAP: community-acquired pneumonia.
N = number of subjects evaluated.
–: Not available.
Values represent the number (%) of subjects.
[a] Calculated as body weight/(height × 0.01)^2.
[b] FAS includes subjects with diseases other than CAP.

Among 102 subjects included in the FAS, 69 subjects had bacterial pneumonia, 9 subjects had atypical pneumonia, and 1 subject had mixed bacterial and atypical pneumonia (Table 2). The remaining 23 subjects with pneumonia did not have the CAP specified in the protocol, 16 of whom did not have CAP such as eosinophilic pneumonia and organizing pneumonia, and 7 subjects were given a diagnosis of CAP but were not eligible for clinical evaluation.

The CPPS included 73 subjects (71.6%). A total of 29 subjects were excluded from the CPPS in reference to the DRC discussion on the conditions that may have an impact on inclusion in the CPPS. The most common primary reasons for exclusion from the CPPS were: subjects who did not have CAP specified in the diagnostic criteria of the protocol such as eosinophilic pneumonia and organizing pneumonia (16 subjects), difficulty of clinical evaluation due to underlying diseases (3 subjects), and inappropriate application of exclusion criteria regarding treatment with systemic antimicrobials prior to the initiation of the study medication (3 subjects). Four different species of causative pathogens were isolated at baseline from 33 of 73 subjects in the CPPS. A single causative pathogen was identified in 26 subjects and multiple causative pathogens were identified in 7 subjects. The most common causative pathogen was *H. influenzae* (17 strains including 1 β-lactamase producing strain and 14 β-lactamase nonproducing strains), followed by *S. pneumoniae* (14 strains), *M. catarrhalis* (5 strains including 4 β-lactamase producing strains) and *M. pneumoniae* (5 strains).

For the subjects in the CPPS, the average administration period of IV AZM was 3.8 days (range from 2 to 5 days) and that of oral AZM was 4.0 days (range from 0 to 7 days) (Table 3). The average treatment period overall was 7.8 days (range from 2 to 10 days). The average length of hospitalization for the CPPS was 8.9 days (range from 3 to 29 days), and that in subjects whose clinical response was assessed as effective on Day 15 by the DRC was 9.4 days (range from 4 to 29 days), which was similar to that in the CPPS overall.

3.2. Efficacy

The results of the clinical response assessed by the DRC (primary efficacy endpoint) are shown in Table 4. The efficacy rate in

Table 2
Details of primary diagnosis.

Primary diagnosis	Analysis set	
	FAS	CPPS
Community-acquired pneumonia	102	73
Bacterial pneumonia	29 (28.4)	28 (38.4)
Single pathogen	23	22
Multiple pathogens	6	6
Suspected bacterial pneumonia	40 (39.2)	36 (49.3)
Chlamydial pneumonia	0	0
Mycoplasma pneumonia	5 (4.9)	4 (5.5)
Legionella pneumonia	0	0
Suspected atypical pneumonia	4 (3.9)	4 (5.5)
Bacterial + atypical pneumonia	1 (1.0)	1 (1.4)
Disease which was not the CAP specified in the protocol	23 (22.5)	–

Abbreviations: FAS, full analysis set; CPPS, clinical per protocol set; CAP, community-acquired pneumonia.
–: Not available.
Values represent the number (%) of subjects.

Table 3
Duration of AZM therapy in the CPPS.

Day of dosing	IV AZM (N = 73)	Oral AZM (N = 73)	IV and oral AZM (N = 73)
0	0	7 (9.6)	0
1	0	1 (1.4)	0
2	3 (4.1)	3 (4.1)	1 (1.4)
3	31 (42.5)	7 (9.6)	3 (4.1)
4	17 (23.3)	20 (27.4)	2 (2.7)
5	22 (30.1)	25 (34.2)	1 (1.4)
6	0	8 (11.0)	2 (2.7)
7	0	2 (2.7)	20 (27.4)
8	0	0	17 (23.3)
9	0	0	10 (13.7)
10	0	0	17 (23.3)
Duration of therapy			
Mean	3.8	4.0	7.8
Min	2	0	2
Max	5	7	10
Median	4.0	4.0	8.0

Abbreviations: AZM, azithromycin; CPPS, clinical per protocol set; IV intravenous.
N = number of subjects evaluated.
Values represent the number (%) of subjects.

the CPPS overall was 84.5% (60/71 subjects) on Day 15 (primary analysis of primary endpoint), 86.3% (63/73) at EOT, and 82.9% (58/70) on Day 29. Efficacy rate was defined as the number of subjects assessed as "effective" divided by the total number of subjects excluding the number of subjects assessed as "indeterminate". The efficacy rates in the FAS were similar to those in the CPPS: 84.9% (62/73) on Day 15, 86.7% (65/75) at EOT, and 83.3% (60/72) on Day 29. The efficacy rate exceeded 80% at all evaluation time points. The clinical response by baseline causative pathogen in the BPPS assessed by the DRC is shown in Table 5. The efficacy rate on Day 15 was 85.7% (12/14) in subjects with *S. pneumoniae*, 88.2% (15/17) in those with *H. influenzae*, 100% (5/5) in those with *M. catarrhalis*, and 100% (4/4) in those with *M. pneumoniae*. The tendency toward clinical improvement on Day 3 was assessed as improved by the investigators in 68 subjects (93.2%) in the CPPS.

For the bacteriological response in the BPPS overall assessed by the DRC, the eradication rate was 71.4% (20/28) on Day 3, 80.6% (25/31) at EOT, 83.3% (25/30) both on Days 15 and 29. The bacteriological response by baseline causative pathogen in the BPPS assessed by the DRC is shown in Table 6. The eradication rate by causative pathogen on Day 15 assessed by the DRC was 85.7% (12/14 strains) for *S. pneumoniae* and 82.4% (14/17 strains) for *H. influenzae*, 100% (5/5 strains) for *M. catarrhalis*, and 100% (2/2 strains) for *M. pneumoniae*.

For the BPPS, of 14 isolates of *S. pneumoniae*, 11 strains were resistant to AZM (MIC ≥2.0 μg/ml), and for 3 isolates, MIC data were not available. The clinical and bacteriological responses by AZM susceptibility of baseline pathogens for subjects in the BPPS

Table 4
Clinical response (DRC assessment, CPPS).

	Total	Clinical response			Efficacy rate[a]	95% CI
		Effective (%)	Ineffective (%)	Indeterminate (%)		
EOT	73	63 (86.3)	10 (13.7)	0	86.3	(76.2, 93.2)
Day 15	71	60 (84.5)	11 (15.5)	0	84.5	(74.0, 92.0)
Day 29	71	58 (81.7)	12 (16.9)	1 (1.4)	82.9	(72.0, 90.8)

Abbreviations: DRC, data review committee; CPPS, clinical per protocol set; CI, confidence interval; EOT, end of treatment.
[a] Efficacy rate = effective/(total − indeterminate) × 100.

Table 5
Clinical response by baseline causative pathogen (DRC assessment, BPPS).

Pathogen[a]	Clinical response					
	EOT		Day 15		Day 29	
	n/N	Efficacy rate[b]	n/N	Efficacy rate[b]	n/N	Efficacy rate[b]
S. pneumoniae	12/14	85.7	12/14	85.7	12/14	85.7
H. influenzae	15/17	88.2	15/17	88.2	15/17	88.2
M. catarrhalis	5/5	100	5/5	100	5/5	100
M. pneumoniae	5/5	100	4/4	100	4/4	100

Abbreviations: DRC, data review committee; BPPS, bacteriologic per protocol set; EOT, end of treatment.
n = number of subjects assessed as effective, and N = number of subjects evaluated excluding those with missing data and those assessed as indeterminate.
[a] A subject may have more than 1 pathogen isolated.
[b] Calculated as $n/N \times 100$.

assessed by the DRCs are shown in Table 7. Of 3 subjects in whom *S. pneumoniae* was isolated and their MIC data were not available. Two cases of pneumococcal pneumonia were diagnosed based on the urinary antigen test without isolation of the pathogen. The clinical responses in both subjects were evaluated as effective. The bacteriological responses in both subjects were evaluated as indeterminate. However, the causative pathogens in both subjects were evaluated as unknown, because *S. pneumoniae* was not obtained in the culture results. Of the subjects in whom AZM-resistant *S. pneumoniae* was isolated, high rates of the clinical efficacy and bacterial eradication were achieved in 10/11 (90.9%). The clinical efficacy and bacterial eradication in 11 subjects in whom AZM-resistant *S. pneumoniae* was identified are summarized in Table 8. Of 11 AZM-resistant *S. pneumoniae* strains, 5 strains showed a relatively low MIC (4–16 μg/ml) and had only the *mef* A gene. The other 6 strains were highly resistant (MIC >64 μg/ml), of which 4 strains had only the *erm* B gene and 2 strains had both the *mef* A and *erm* B genes. Even for high-level resistant *S. pneumoniae* strains (MIC >64 μg/ml) consisting of 4 strains with only the *erm* B gene and 2 strains with both the *mef* A and *erm* B genes, the efficacy and eradication rates were 100% (6/6). AZM treatment failed in only 1 subject with CAP caused by AZM-resistant *S. pneumoniae* (MIC = 8 μg/ml with the *mef* A gene at all evaluation time points. The subject showed persistence of fever, increased CRP, and an aggravated filtrate in chest X-ray findings from Day 3 onwards. AZM IV-to-oral therapy was effective for CAP in 10 out of the 11 subjects (90.9%), regardless of high-level macrolide-resistance *in vitro* and macrolide resistance gene development of *S. pneumoniae* strains. Fig. 1 shows significantly improved clinical signs and symptoms, and chest X-ray findings in 2 cases with CAP caused by highly resistant *S. pneumoniae* strains (MIC >64 μg/ml) with both the *mef* A and *erm* B genotypes.

For subjects in the BPPS in whom susceptible *H. influenzae* strains (MIC ≤4 μg/ml) were identified, the efficacy rate was 86.7% (13/15) at all assessment time points, while the eradication rate was 80.0 (12/15) to 93.3% (14/15). All of the 17 isolates of *H. influenzae* were susceptible to AZM (MIC ≤4 μg/ml) except 2 isolates for which MIC data were not available.

Clinical signs and symptoms (cough, sputum volumes, nature of sputum, dyspnea, consciousness disturbed, chest pain, chest rales, dehydration, and pain pharyngeal) in subjects in the CPPS tended to improve from Day 3 onwards. Body temperature, WBC count, CRP, and SpO_2 improved in most of the subjects after switching to the oral compared to the values observed on Day 1 (prior to dosing) as shown in Fig. 2. The pulmonary infiltrates on chest X-ray score improved from Day 3 onwards, and further improved over the therapeutic course in most subjects.

Table 6
Bacteriological response by baseline causative pathogen (DRC assessment, BPPS).

Pathogen[a]	Bacteriological response							
	Day 3		EOT		Day 15		Day 29	
	n/*N*	Eradication rate[b]	*n*/*N*	Eradication rate[b]	*n*/*N*	Eradication rate[b]	*n*/*N*	Eradication rate[b]
S. pneumoniae	6/13	46.2	9/14	64.3	12/14	85.7	12/14	85.7
H. influenzae	14/15	93.3	16/17	94.1	14/17	82.4	14/17	82.4
M. catarrhalis	5/5	100	5/5	100	5/5	100	5/5	100
M. pneumoniae	3/3	100	3/3	100	2/2	100	2/2	100

Abbreviations: DRC, data review committee; BPPS, bacteriologic per protocol set; EOT, end of treatment.
n = number of pathogens with eradication or presumed eradication, and *N* = total number of pathogens excluding the number of pathogens with indeterminate.
[a] A subject may have more than 1 pathogen isolated.
[b] Calculated as $n/N \times 100$.

The medical rationales for switching from IV to oral therapy for subjects in the CPPS were improvement in clinical signs and symptoms (body temperature, WBC count, and CRP) in 93.9% of subjects (62/66) and improvement in clinical symptoms (other than body temperature, WBC count, and CRP) in 6.1% of subjects (4/66). IV AZM therapy was switched to oral AZM between Day 2 and Day 5 (an average IV AZM administration period, 3.8 days) in the CPPS. The descriptive statistics (median, 75th and 90th percentiles) for body temperature, WBC count, and CRP at the time of switching from IV to oral therapy are shown in Fig. 2. The therapy was switched from IV to oral AZM administration by approximately 75% of the investigators for the subjects with the body temperature under 37°C, the WBC counts under 8600/mm^3, or the CRP under 7.5 mg/dl.

3.3. *Safety results*

Among the 102 subjects, 55 subjects (53.9%) experienced all-causality adverse events during the entire period. Treatment-related adverse events were observed in 34 subjects (33.3%) during the entire period.

The most common all-causality adverse events were diarrhea (15 subjects, 14.7%), constipation (8 subjects, 7.8%), headache (8 subjects, 7.8%), injection site pain (6 subjects, 5.9%), and insomnia (6 subjects, 5.9%). Most adverse events were mild to moderate in severity. Two severe adverse events included pneumonia in 1 subject (investigator term, underlying disease aggravated) and lung malignant neoplasm in 1 subject, which were non-treatment-related, serious adverse events and led to study discontinuation.

Seven subjects (6.9%) discontinued the study or the study medication due to adverse events. Of these adverse events, diarrhea and abdominal pain in 1 subject each were considered to be treatment-related adverse events, which were confirmed to have resolved.

No subject died in this study. Ten serious adverse events were observed in 9 subjects. The only serious adverse event considered by the investigator to be treatment-related adverse event was prothrombin time prolonged (mild in severity) in 1 subject, which was confirmed to have resolved.

4. Discussion

In this study, AZM IV-to-oral switch therapy demonstrated excellent clinical efficacy and bacterial eradication in the treatment of moderate to severe CAP regardless of the type and *in vitro* susceptibility of the causative pathogens including *S. pneumoniae*, which is the most commonly identified pathogen for CAP worldwide as well as in Japan [18]. Even for high-level resistant *S. pneumoniae* strains (MIC >64 μg/ml) consisting of 4 strains with the *erm* B gene and 2 strains with both the *mef* A and *erm* B genes, the clinical efficacy and eradication rates were 100% (6/6).

A number of studies have indicated the discordance between increasing resistance *in vitro* and clinical effectiveness of the macrolide therapy against macrolide-resistant *S. pneumoniae*

Table 7
Clinical and bacteriological responses by AZM MIC of baseline causative pathogen (DRC assessment, BPPS).

Pathogen[a]	MIC	EOT		Day 15		Day 29	
		Efficacy rate[b] (%)	Eradication rate[c] (%)	Efficacy rate[b] (%)	Eradication rate[c] (%)	Efficacy rate[b] (%)	Eradication rate[c] (%)
S. pneumoniae (Total)		12/14 (85.7)	9/14 (64.3)	12/14 (85.7)	12/14 (85.7)	12/14 (85.7)	12/14 (85.7)
	MIC ≥2 μg/ml	10/11 (90.9)	7/11 (63.6)	10/11 (90.9)	10/11 (90.9)	10/11 (90.9)	10/11 (90.9)
	MIC unknown	2/3 (66.7)	2/3 (66.7)	2/3 (66.7)	2/3 (66.7)	2/3 (66.7)	2/3 (66.7)
H. influenzae (Total)		15/17 (88.2)	16/17 (94.1)	15/17 (88.2)	14/17 (82.4)	15/17 (88.2)	14/17 (82.4)
	MIC ≤4 μg/ml	13/15 (86.7)	14/15 (93.3)	13/15 (86.7)	12/15 (80.0)	13/15 (86.7)	12/15 (80.0)
	MIC unknown	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)
M. catarrhalis (Total)		5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
	MIC ≤0.03 μg/ml	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
	MIC = 0.06 μg/ml	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
	MIC unknown	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
M. pneumoniae (Total)		5/5 (100)	3/3 (100)	4/4 (100)	2/2 (100)	4/4 (100)	2/2 (100)
	MIC = 0.00025 μg/ml	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)
	MIC = 0.005 μg/ml	1/1 (100)	1/1 (100)	0 (–)	0 (–)	0 (–)	0 (–)
	MIC unknown	2/2 (100)	–	2/2 (100)	–	2/2 (100)	–

Abbreviations: AZM, azithromycin; DRC, data review committee; BPPS, bacteriologic per protocol set; EOT, end of treatment.
–: Not available.
[a] A subject may have more than 1 pathogen isolated.
[b] Efficacy rate = effective/(total − indeterminate) × 100.
[c] Eradication rate = (eradication + presumed eradication)/(total − indeterminate) × 100.

Table 8
Clinical and bacteriological responses in 11 patients in whom AZM-resistant *S. pneumoniae* was isolated.

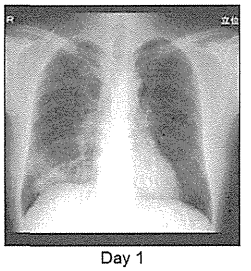
In vivo											In vitro					
Case No.	Age	Sex	CAP severity	Body temperature (°C)		WBC (/mm^3)		CRP (mg/dl)		Clinical efficacy	Bacteriological response	Genotype	MIC (μg/ml)				Mixed infection
				Day 1	Day 15	Day 1	Day 15	Day 1	Day 15	Day 15	Day 15		AZM	PCG	CPFX	LVFX	
1	77	F	Moderate	37.6	35.1	17,300	7800	28.30	0.30	Cure	Eradication	mef A	16	0.06	1	1.0	*H. influenzae*
2	56	F	Moderate	38.5	36.9	17,500	4500	24.90	0.20	Cure	Eradication	mef A	16	1	0.5	0.5	No
3	78	M	Moderate	38.8	36.3	11,300	4800	3.40	0.60	Cure	Presumed eradication	mef A	4	0.06	1.0	1.0	No
4	60	M	Moderate	39.4	37.0	10,500	7200	9.10	0.00	Cure	Presumed eradication	erm B	>64	2	0.5	0.5	*M. catarrhalis*
5	55	M	Moderate	38.4	36.3	16,400	7100	8.80	0.40	Cure	Presumed eradication	mef A + erm B	>64	0.12	1.0	1.0	No
6	23	F	Moderate	38.6	36.3	16,300	6000	16.80	0.10	Cure	Presumed eradication	erm B	>64	0.06	1.0	1.0	No
7	38	M	Moderate	39.4	36.8	11,040	4980	4.31	0.04	Cure	Presumed eradication	erm B	>64	≤0.03	1.0	1.0	No
8	25	M	Moderate	37.5	36.4	10,500	4000	4.80	0.00	Cure	Presumed eradication	mef A	16	0.06	1.0	1.0	No
9	24	F	Moderate	38.0	36.5	16,800	7900	4.40	0.00	Cure	Presumed eradication	erm B	>64	0.25	1.0	0.5	*H. influenzae*
10	42	F	Moderate	39.5	38.8 (EOT)	32,400	10,200 (EOT)	13.90	17.30 (EOT)	Failure	Persistence	mef A	8	≤0.03	2.0	1.0	No
11	71	M	Moderate	38.5	36.8	4800	4700	10.90	0.10	Cure	Eradication	mef A + erm B	>64	≤0.03	0.5	0.5	*M. pneumoniae*

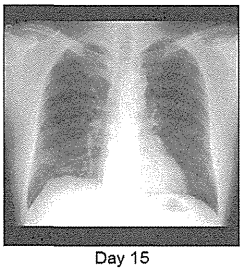
Abbreviations: AZM, azithromycin; CAP, community-acquired pneumonia; WBC, white blood cell; PCG, penicillin G; CPFX, ciprofloxacin; LVFX, levofloxacin; F, female; M, male; mef, macrolide efflux pump; erm, erythromycin ribosomal methylase; EOT, end of treatment.

in vivo [10,12–15]. The results in the present study also show the discrepancy between clinical efficacy of AZM IV-to-oral switch therapy against *S. pneumoniae in vivo* and low susceptibility to AZM *in vitro*. This "*in vivo-in vitro* paradox" may be explained by the following unique properties of AZM. AZM has unique pharmacological properties such as phagocyte delivery and extremely long half-life. The concentrations of AZM in WBC were more than 100 times higher than those in plasma following 500 mg IV AZM for 3 days, and the exposure to WBC for 3 days was estimated more than 1000 times greater than that to plasma [19]. $AUC_{0\text{-}24}$ of AZM in epithelial lining fluid and alveolar macrophage were 45.8 and 14,944 μg·h/ml respectively, which indicated 5.6 and 1800 times higher than the value in plasma following 500 mg IV AZM for 5 days [20]. Another unique property of AZM which might be related to the effectiveness of AZM against macrolide-resistant *S. pneumoniae* is the inhibitory effect on pneumolysin production of *S. pneumoniae in vitro* and *in vivo* [21]. Sub-MIC of AZM inhibited pneumolysin production and activity of *S. pneumoniae in vitro*, and oral administration of AZM (200 mg/kg) reduced pneumolysin in the lungs in mice infected with *S. pneumoniae* and improved the survival rates *in vivo*. Macrolides including AZM were reported to have anti-inflammatory and immunomodulatory activities both *in vitro* and *in vivo*, which may have a favorable effect on the clinical outcome of patients with CAP by controlling the exacerbation of underlying respiratory problems, such as cystic fibrosis, asthma, bronchiectasis, panbrochiolitis and cryptogenic organizing pneumonia [22]. The discrepancy between clinical efficacy of AZM treatment and *in vitro* resistance suggests that the resistance criteria and breakpoint of AZM against *S. pneumoniae* can be revised.

Case: 5

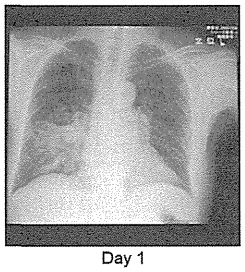
Clinical Symptom	Evaluation Date				
	Day 1	Day 3	EOT	Day 15	Day 29
Cough	1+	1+	-	-	-
Sputum Volumes	1+	1+	-	-	-
Nature of Sputum*	PM	PM	-	-	-
Chest Rales	+	+	-	-	-
Body Temperature (°C)	38.4	36.7	36.5	36.3	36.4
WBC (/mm^3)	16,400	9,800	7,300	7,100	7,000
CRP (mg/dl)	8.80	10.80	0.90	0.40	0.40


Day 1


Day 15

Case: 11

Clinical Symptom	Evaluation Date				
	Day 1	Day 3	EOT	Day 15	Day 29
Cough	3+	1+	2+	1+	1+
Sputum Volumes	2+	2+	2+	1+	-
Nature of Sputum*	P	P	M	M	-
Chest Rales	+	-	-	-	-
Body Temperature (°C)	38.5	36.6	36.6	36.8	36.7
WBC (/mm^3)	4,800	4,200	6,100	4,700	3,700
CRP (mg/dl)	10.90	7.50	1.10	0.10	0


Day 1

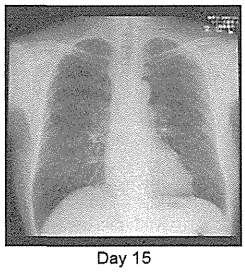

Day 15

Fig. 1. Clinical signs and symptoms, and chest X-ray findings in 2 cases with CAP due to highly resistant *S. pneumoniae* strains (MIC >64 μg/ml) with both the *mef* A and *erm* B genotypes. Abbreviations: EOT, end of treatment; PM, mucopurulent; P, purulent; M, mucoid; WBC, white blood cell; CAP, community-acquired pneumonia; erm, erythromycin ribosomal methylase; mef, macrolide efflux pump.

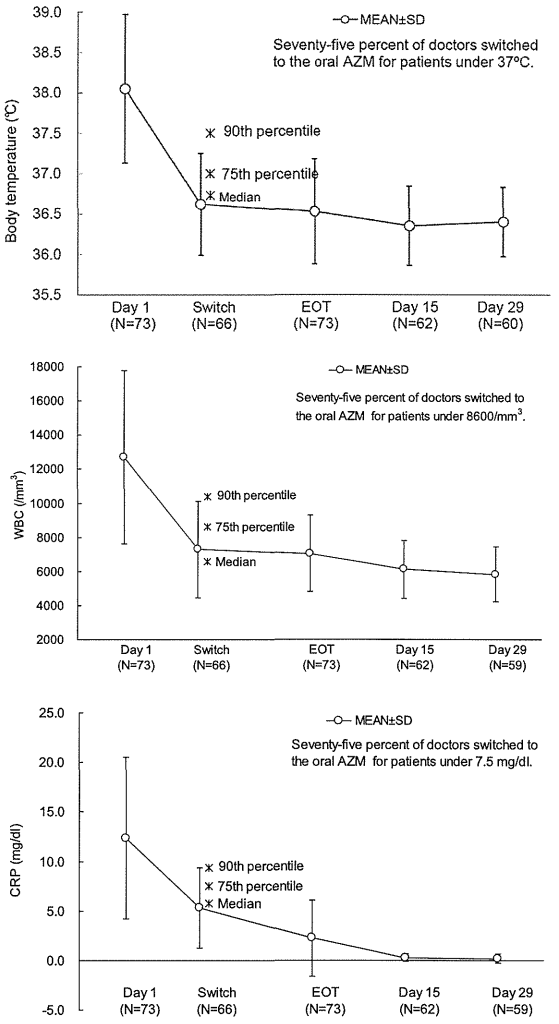


Fig. 2. Time course of body temperature, WBC count, and CRP for subjects in the CPPS. The descriptive statistics (median, 75th and 90th percentiles) of these parameters at the time of switching from IV to oral therapy are indicated in the figure. Abbreviations: AZM, azithromycin; EOT, end of treatment; WBC, white blood cell; CPPS, clinical per protocol set; IV, intravenous.

Many studies have provided evidence that macrolide resistance is clinically relevant to the clinical outcome in the treatment of pneumococcal pneumonia, indicating that macrolide resistance is an emerging problem in the empirical treatment of macrolide-resistant *S. pneumoniae* [9–12,23]. Although clinical constraints make it difficult to demonstrate the clinical impact of macrolide resistance in CAP, increasing resistance in *S. pneumoniae* obviously provide numerous clinical challenges for the empirical treatment of pneumococcal pneumonia as indicated in these studies. Therefore, it is important to choose the effective treatment strategies such as AZM IV-to-oral switch therapy as demonstrated in this study.

H. influenzae and M. pneumoniae were also identified as causative pathogens in this study. *C. pneumoniae*, however, was not detected, even though it is one of the top 4 pathogens involved in CAP both in Japan and the United States of America [2,4].

In this study, IV AZM therapy was switched to oral AZM therapy between Day 2 and Day 5 in CPPS, with an average IV AZM treatment period of 3.8 days. Most subjects showed improvement in clinical signs and symptoms (body temperature, WBC count, and CRP) at the time of switching to oral therapy, and the eradication rate for subjects in the BPPS was 71.4% (20/28) on Day 3. The average length of hospitalization for subjects in the CPPS was 8.9 days (range from 3 to 29 days). In CPPS, 60 subjects were considered effective at Day 15 by the DRC assessment. Among 60 subjects, 46 subjects were hospitalized. They were discharged from the hospital (1) by the first day of oral administration (8 subjects, 17.4%), (2) between the second day of oral administration and the first day after EOT (27 subjects, 58.7%), (3) between the second day after EOT and the 16th day after the start of the treatment (8 subjects, 17.4%), and (4) on and after the 17th day after the start of the treatment (3 subjects, 6.5%). These results together with that the tendency toward clinical improvement on Day 3 assessed as improved by the investigators in most CPPS subjects (93.2%) indicate most subjects continued to receive inpatient care even after improvement of clinical symptoms and switching to oral AZM. It appeared that the investigators actually determined the timing of patient discharge considering not only these parameters, but also the individual patient's social conditions. IV-to-oral switching therapy is considered to have an advantage to shorten the patient's hospitalization. Ambulatory treatment with injectable antibacterial agents requires patients to receive injection at the hospital, but treatment with oral reduces the restraint of activity and the burden on patients because patients do not need to go to the hospital frequently, leading to an early return to normal life. Shorter hospital stays and cost effectiveness of early switching to oral antibacterial therapy for CAP were reported in the study of transition to oral cefaclor therapy after an abbreviated course of IV cefamandole therapy [24].

The current study has some limitations. Firstly, the current clinical trial was not specifically targeted to investigate the efficacy of azithromycin against pneumococcal pneumonia. In addition, AZM-resistant *S. pneumoniae* was isolated in only 11 cases. Therefore, it is necessary to further investigate pneumococcal pneumonia in particular. Secondly, since Chlamydial pneumonia was not detected, it was not possible to evaluate the clinical efficacy of AZM on Chlamydial pneumonia in this study. Thirdly, enrollment in this study was limited to subjects who met the protocol-specified inclusion criteria to evaluate the clinical efficacy and safety of AZM in a well-defined patient population appropriate for Japanese regulatory approval. In particular, the Japanese evaluation guidance for antimicrobial agents was used to define the inclusion criteria regarding the severity of pneumonia. It is noted that these criteria are not identical with the international standards of pneumonia severity such as the pneumonia severity index (PSI), CURB-65, and the A-DROP system. Therefore caution is required when interpreting the current study results in the context of international severity standards. It is also noted that in actual medical practice, IV AZM is administered to more severe patients than those in the current study, partly due to the recommendations in the IDSA/ATS consensus guidelines on the management of CAP in adults [4].

A recent study reported that intubated patients with severe CAP admitted to the ICU who received combination therapy with macrolides plus β-lactams had a significantly lower mortality rate than those who received combination therapy with quinolones plus β-lactams (26.1% [12/46] vs. 46.3% [25/54]) [25]. Furthermore, a systematic review and meta-analysis on macrolide-based treatment regimens and mortality in the hospitalized patients with CAP reported that the macrolide-based regimens were associated with a significant mortality reduction compared with non-macrolides [26]. These findings indicate that guideline concordant antimicrobial therapy is important for the initial empirical treatment of CAP.

In conclusion, AZM IV-to-oral switch therapy demonstrated excellent clinical and bacteriological effects on moderate-to-severe

pneumococcal pneumonia despite a high MIC and resistance gene development. This discrepancy is referred to as the "*in vivo-in vitro* paradox". The current study results provide an insight into this paradox.

Conflict of interest

S. Kohno, K. Tateda, J. Kadota, J. Fujita, Y. Niki, and A. Watanabe have received consultant fees from Pfizer Japan Inc., and M. Nagashima is an employee of Pfizer Japan Inc.

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Original article

Clinical evaluation of high mobility group box 1 protein in *Legionella pneumophila* pneumonia

Futoshi Higa*, Makoto Furugen, Michio Koide, Yosuke Karimata, Daijiro Nabeya, Yoshikazu Iha, Takeshi Kinjo, Kazuya Miyagi, Shusaku Haranaga, Akira Hokama, Masao Tateyama, Jiro Fujita

Department of Infectious, Respiratory, and Digestive Medicine, Graduate School of Medicine, University of the Ryukyus, 903-0215 Okinawa, Japan

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ABSTRACT

High mobility group box 1 (HMGB-1) protein is involved in acute lung injury due to various etiologies. We evaluated HMGB-1 levels in sera and bronchoalveolar fluids in patients with pneumonia caused by *Legionella pneumophila*. Levels of HMGB-1 in the sera of patients with *L. pneumophila* pneumonia (32 cases) and control subjects (24 cases) were determined. Serum HMGB-1 levels in *Legionella* pneumonia were similar to those of the control subjects. No significant correlation between HMGB-1 levels and other biomarkers and the outcome of cases was observed. In contrast, HMGB-1 levels, as well as interferon-γ, in bronchoalveolar (BA) fluids from severe *L. pneumophila* pneumonia (7 cases) were significantly higher than those in the sera of identical patients. HMGB-1 levels in BA fluids were relatively higher in pneumonia cases with ALI than those without ALI. Our findings suggest that intra-pulmonary HMGB-1 may be involved in the pathophysiology of pneumonia caused by *L. pneumophila*.

1. Introduction

Bacteria of the genus *Legionella* are important causative agents of epidemic and sporadic pneumonia in humans [1]. The most common species in the genus is *Legionella pneumophila. Legionella* species is an important pathogen causing severe community-acquired and hospital-acquired pneumonia requiring intensive care unit admission [2–4]. *Legionella* pneumonia progresses rapidly and is often complicated by acute lung injury/acute respiratory distress syndrome (ALI/ARDS) [5,6].

The high mobility group box 1 (HMGB-1) protein belongs to the nuclear nonhistone protein family, which is involved the transcription of genes within the nucleus [7]. Recent studies have shown that the HMGB-1 protein is released from activated macrophages and dying cells. The released extracellular HMGB-1 works as an inflammatory cytokine and is involved in the late phase of sepsis [8] and ALI/ARDS [9]. HMGB-1 was also released from alveolar epithelial cells and alveolar macrophages when they were infected with *L. pneumophila* [10,11], suggesting that this cytokine is involved in the pathophysiology of *Legionella* pneumonia. However, the clinical significance of HMGB-1 in the disease remains unclear.

In this study, we examined the concentrations of HMGB-1 in the sera and bronchoalveolar (BA) fluids of patients with *L. pneumophila* pneumonia to evaluate the clinical role of HMGB-1 in *Legionella* pneumonia.

2. Materials and methods

2.1. Study population

Thirty-six consecutive cases with *L. pneumophila* pneumonia diagnosed in our laboratory from 1997 through 2007 were included in this study. Four cases were excluded because of a lack of appropriate samples. Blood samples were obtained for the conventional clinical diagnosis of the patients. BA fluids were obtained with written informed consent when required to diagnose *Legionella* pneumonia. The samples were collected when the diagnosis of *Legionella* pneumonia was established. These samples were stored at −80 °C until further use. Medical chart reviews were used to obtain information regarding the laboratory findings and clinical outcome for each patient. Severity of pneumonia was determined using pneumonia severity index [12].

* Corresponding author. Department of Infectious, Respiratory, and Digestive Medicine, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. Tel.: +81 98 895 1144; fax: +81 98 895 1414.
E-mail address: fhiga@med.u-ryukyu.ac.jp (F. Higa).

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This study was approved by the University of the Ryukyus Institutional Review Board. The need for informed consent from each patient for inclusion in this study was waived because this study was retrospective in approach, which caused no additional adverse events in any subject.

2.2. Diagnosis of Legionella pneumonia

Pneumonia was diagnosed based on clinical presentation (symptoms and physical examination), chest X-ray findings, and laboratory data. The diagnosis of *Legionella* pneumonia was confirmed by detecting *Legionella* in culture, the elevation of antibody titers in paired sera, and/or the detection of its specific antigen in the urine. Control subjects had no known infectious disease, liver disease, or kidney disease.

2.3. Determination of HMGB-1 and other biomarkers

The HMGB-1 concentration in each sample was determined via a sandwich ELISA (Shino-Test Co., Tokyo, Japan) using recombinant swine HMGB-1 as a standard [13]. The detection limit of HMGB-1 was 0.1 ng/mL. Hepatocyte growth factor (HGF) and interferon-gamma (IFN-γ) levels in each sample were determined using a sandwich ELISA (R&D Systems, Minneapolis, MN, USA). The lowest detection limits for HGF and IFN-γ were 40 pg/mL and 8 pg/mL, respectively. The albumin concentration in each sample was determined using a QuantiChrom BCG albumin assay kit (BioAssay Systems, Hayward, CA, USA).

3. Statistical analysis

Differences between groups were analyzed using the unpaired *t*-test, Fischer's exact probability test, the Mann–Whitney *U* test, or the Wilcoxon matched-pairs signed rank test using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The association between biomarkers was evaluated by Pearson's correlation coefficient using the IBM SPSS statistics version 21 (SPSS, Inc., Chicago, IL, USA).

4. Results

The average age of the 32 patients with *Legionella* pneumonia was 59.8 ± 11.1 years; this group included 28 men and 4 women. The average of age of the 24 control subjects was 54.2 ± 19.1 years. These subjects included 17 men and 7 women. Statistical analyses of age (unpaired *t*-test) and sex (Fischer's exact probability test) between groups showed no significant differences. The serum HMGB-1 concentrations for patients and control subjects were determined. The serum HMGB-1 levels of the *L. pneumophila* pneumonia patients (mean ± SD; 5.8 ± 6.4 ng/mL) were similar to those of the healthy control subjects (mean ± SD; 4.6 ± 3.2 ng/mL) (Fig. 1). No significant linear associations between serum HMGB-1 and other biomarkers (white blood cell counts, C-reactive protein, lactate dehydrogenase) and oxygenation parameters (PaO_2 and PaO_2/FiO_2) were observed (Table 1). Additionally, Serum HMGB-1 levels did neither differ between pneumonia cases with different pneumonia severity index [12] (Table 2) nor between 24 survivors (mean ± SD; 5.8 ± 6.8 ng/mL) and 8 non-survivors (mean ± SD; 5.6 ± 5.0 ng/mL).

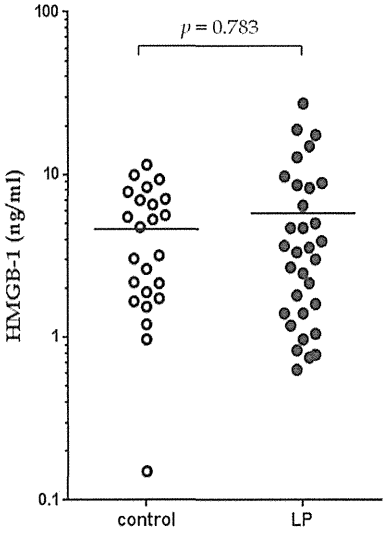

Fig. 1. Serum concentration of HMGB-1 in patients with *Legionella pneumophila* pneumonia and healthy control subjects. HMGB-1 levels were determined using a sandwich ELISA. No significant difference was observed between the 2 groups (Mann–Whitney *U* test).

Next, we determined the HMGB-1 levels in the BA fluids of 7 patients with *Legionella* pneumonia. The HMGB-1 levels in the BA fluids were significantly higher than those in the sera of identical patients (Fig. 2a). The HMGB-1 levels in the BA fluids of patients with ALI (PaO_2/FiO_2 ratio ≤ 200) were relatively higher than those in patients without ALI (PaO_2/FiO_2 ratio > 200) (Fig. 2b). Similar results were observed when HMGB-1 concentrations were normalized using albumin concentration (data not shown). IFN-γ concentrations were also higher in BA fluids than in sera of identical patients, while HGF concentrations were similar in sera and BA fluids (Fig. 2c and d).

5. Discussion

This study revealed that serum HMGB-1 levels of *Legionella* pneumonia were similar to those in healthy control subjects. This finding disagrees with the results of previous studies in which serum/plasma HMGB-1 was higher in community-acquired infections and pneumonia than in healthy subjects [14–16]. In these reports, the average serum HMGB-1 in control subjects was lower

Table 1
Association between serum cytokine concentrations and various serum biomarkers and the oxygenation index.

	Pearson's correlation coefficient (*p* value)				
	WBC	LDH	CRP	PaO_2	PaO_2/FiO_2
HMGB-1	−0.021 (0.911)	0.089 (0.641)	0.309 (0.103)	0.045 (0.813)	0.118 (0.535)
HGF	−0.149 (0.393)	0.517 (0.002)*	0.058 (0.753)	−0.066 (0.717)	−0.077 (0.669)
IFN-γ	−0.176 (0.370)	0.012 (0.952)	0.036 (0.864)	−0.080 (0.944)	0.346 (0.077)

HMGB-1: high mobility group box 1 protein, HGF: hepatocyte growth factor, IFN-γ: interferon-gamma, WBC: white blood cell counts, LDH: lactate dehydrogenase, CRP: C-reactive protein, PaO_2: Arterial oxygen pressure.
Asterisks represent significant associations.

Table 2
Association between pneumonia severity index and serum HMGB-1.

PSI risk class	Cases	Mortality	Serum HMGB-1 Mean ± SD	
2	2	0	4.46[a]	7.29 ± 8.96[b]
3	6	0	8.24 ± 10.33[a]	
4	17	2	5.00 ± 5.52[a]	5.28 ± 5.38[b]
5	7	6	5.96 ± 5.37[a]	

PSI: Pneumonia severity index [12], HMGB-1: high mobility group box 1 protein.
[a] No statistical difference between each group (Mann–Whitney *U* test).
[b] No statistical difference between each group (Mann–Whitney *U* test).

than the value determined for control subjects in the present study. A recent report showed that the median and interquartile range of the serum HMGB-1 levels of healthy subjects were 5.4 ng/mL and 4.0–6.7 ng/mL [17], respectively, which were similar to those of the control subjects in the present study (median: 5.2 ng/mL, interquartile range: 3.7–6.7 ng/mL). Circulating HMGB-1 increases in various non-infectious diseases, such as malignancy [17,18], acute liver injury [19], and chronic kidney disease [20]. Defining normal healthy subjects may be difficult, particularly using HMGB-1, and selection bias can result in differences between the various studies. However, the present study suggests that serum HMGB-1 may not elevate in *Legionella* pneumonia. Future studies should examine whether this finding is specific to pneumonia due to *Legionella*.

HMGB-1 levels were increased in the BA fluids compared to those in the sera of *Legionella* pneumonia patients. In addition, HMGB-1 levels in the BA fluids were relatively higher in pneumonia cases with ALI ($PaO_2/FiO_2 \leq 200$) than in those without ALI. This finding is in agreement with a previous report, in which HMGB-1 increased in lung epithelial lining fluids and in BA lavage fluids of ALI patients [9]. Intra-pulmonary HMGB-1 production contributes to ALI [9] and ventilator-induced lung injury [21]. Thus, a high HMGB-1 concentration in the lungs may be associated with ALI development in *Legionella* pneumonia patients. The source of HMGB-1 may be alveolar epithelial cells [10] and/or alveolar macrophages [11]. It is conceivable that intra-pulmonary HMGB-1 is also involved in severe pneumonia caused by other pathogens, which should be examined in further studies.

Previous studies showed that HGF and IFN-γ are increased in the sera of patients with *Legionella* pneumonia [22,23] and that these cytokines may be useful biomarkers of pneumonia. Therefore, we determined intra-pulmonary HGF and IFN-γ concentrations, and found that IFN-γ increased in the lung more than in the sera of pneumonia cases while HGF concentrations in the lung were similar to the serum HGF levels. HGF functions in the repair of the

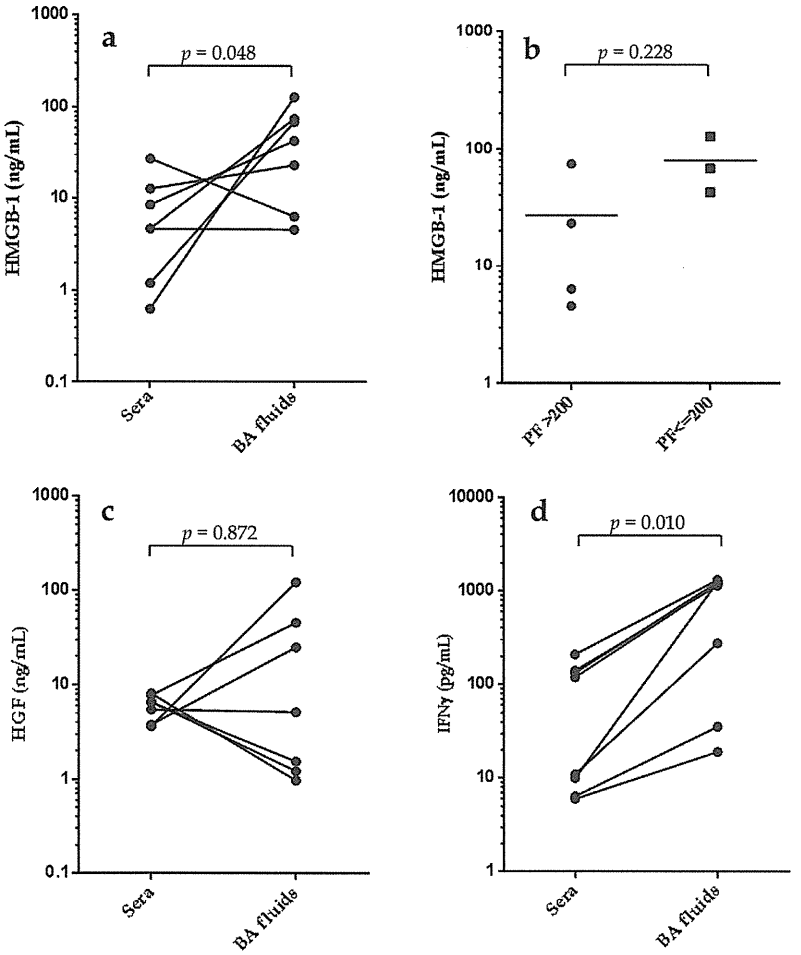

Fig. 2. Concentrations of cytokines in the sera and bronchoalveolar fluids of *Legionella pneumophila* pneumonia. a: high mobility group box 1 protein (HMGB-1), b: HMGB-1 in bronchoalveolar fluids of pneumonia with acute lung injury and without acute lung injury. c: hepatocyte growth factor, d: interferon-gamma. Statistical differences were determined by using Mann–Whitney *U* test.