developed an EIA kit detecting serum IgA antibody specific for GPL core and investigated its usefulness in a multicenter study.

## METHODS

*See* the online supplement for additional methodologic details.

### Patients and Serum Samples

Six institutions participated in this study. Between June 2003 and December 2005, serum samples were collected from 70 patients with MAC-PD, 18 with MAC contamination, 36 with pulmonary TB, 45 with other lung diseases, and 76 healthy subjects. All patients with MAC-PD met the ATS guidelines (1). Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy for mycobacterial diseases recommended by the ATS guidelines, but had MAC-positive cultures at the time of serum collection. Pulmonary TB was confirmed by culture positivity for *M. tuberculosis*. Patients with pulmonary TB who had an underlying pulmonary disease or past history of treatment for pulmonary TB were excluded. Individuals with MAC contamination showed a single culture positive for MAC in small amounts, but were asymptomatic and had no significant chest computed tomography (CCT) findings indicating active mycobacterial disease. The other lung diseases included chronic obstructive pulmonary disease (n = 15), idiopathic interstitial pneumonia (n = 11), lung cancer (n = 11), bacterial pneumonia (n = 4), pulmonary sarcoidosis (n = 2), and bronchiectasis (n = 2). All sera were stored at −20°C until assayed for IgA GPL core antibody. None of the patients was seropositive for HIV type 1 or 2. The patients with MAC-PD were classified into two groups on the basis of the chest radiography: fibrocavitary disease and nodular-bronchiectatic (NBE) disease (1).

Fibrocavitary disease was defined as the presence of cavitary forms in upper lobes. NBE disease was defined as the presence of bronchiectasis and multiple nodular shadows on CCT. Disease conforming to neither of these types was considered unclassifiable. Forty-five patients underwent CCT and serodiagnosis at the same time. A correlation between the extent of disease and antibody levels was investigated. The extent of disease was expressed as the number of MAC-involved CCT segments, as described in the previous study (9).

The studies in human subjects were approved by the research and ethical committees of the NHO National Toneyama Hospital, and written, informed consent was obtained from all subjects.

### EIA Kit

The EIA kit was developed by Tauns Laboratories, Inc. (Shizuoka, Japan), with a slight modification of the method described previously (8). Results are given as arbitrary U/ml in relation to a standard curve that was constructed by mixing sera from three patients with MAC-PD as a reference. The intra- and interplate coefficients of variation were 2.27–9.29% and 0.57–8.86%, respectively, which indicated good reproducibility. The linearity of measurement was confirmed. The influence of blood elements and temperature was examined, and revealed good stability. The assay was performed by a technologist with no prior knowledge of the clinical data.

### Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA). Antibody levels in patient groups are expressed as means ± SD. For comparison of the mean values of multiple groups, data were compared by analysis of variance and nonparametric analysis. A probability value of less than 0.05 was regarded as significant.

## RESULTS

### Study Subjects

The characteristics of the subjects are shown in Table 1. Patients with pulmonary TB and healthy subjects were younger than patients with MAC-PD ($P < 0.001$), and there was a larger proportion of females in the latter group ($P < 0.001$). Of the 70 patients with MAC-PD, 15 had underlying pulmonary disease, all of which were the sequelae of pulmonary TB. Of the 18 individuals with MAC contamination, 15 had underlying pulmonary diseases (8 patients with the sequelae of pulmonary TB, 2 with lung cancer, 2 with chronic obstructive pulmonary disease, 1 with emphysema, 1 with pneumoconiosis, and 1 with sarcoidosis. Of the patients with MAC-PD, 19 were classified as having fibrocavitary disease, and 35 as having NBE disease, with 16 patients unclassifiable. The MAC-PD group included infections with *M. avium* (n = 56), *Mycobacterium intracellulare* (n = 12), or both (n = 2). The MAC contamination group included *M. avium* (n = 16) and *M. intracellulare* (n = 2).

### Level of GPL Core IgA Antibody

The level of serum IgA antibody to GPL core was quantified using the EIA kit (Figure 1). As expected, patients with MAC-PD had significantly higher levels than patients with MAC contamination, those with pulmonary TB, those with other lung diseases, and healthy subjects—namely, 10.7 ± 7.9, 0.2 ± 0.1, 0.1 ± 0.1, 0.0 ± 0.1, and 0.0 ± 0.0 U/ml, respectively ($P < 0.0001$). A receiver operating characteristic (ROC) curve was constructed for MAC-PD and the other groups to establish the best cutoff value (Figure 2). Setting the cutoff value at 0.7 U/ml resulted in 100% specificity, at a sensitivity of 84.3% (Table E1). Using the EIA kit allowed clear discrimination between patients with MAC-PD and MAC contamination, pulmonary TB, and other lung diseases, as well as healthy subjects.

Next, we compared levels of serum IgA antibody to GPL core in fibrocavitary disease and NBE disease of MAC-PD. Significantly higher levels were found in NBE ($P < 0.05$) (Figure 3). With the cutoff value set at 0.7 U/ml, positivity in NBE and fibrocavitary disease was 91.4 and 63.2%, respectively. In contrast, in patients with MAC-PD, no significant differences between *M. avium* and *M. intracellulare* as causative agents were observed ($P = 0.403$). The erythrocyte sedimentation rate in MAC-PD was 32.6 ± 28.6 mm/hour and there was a significant positive correlation between the erythrocyte sedimentation rate and antibody levels in patients with MAC-PD ($r = 0.294$, $P < 0.05$).

### Radiographic Severity and the Level of GPL Core Antibody

Forty-five patients with MAC-PD (10 with fibrocavitary disease, 26 with NBE disease, and 9 with unclassifiable type disease) underwent CCT and serodiagnosis at the same time. Four patients with unclassifiable type disease were excluded from the investigation because it was hard to discriminate between MAC lesions and underlying pulmonary disease. There was a positive correlation between the extent of disease and the

**TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS**

| | MAC-PD | MAC Contamination | Pulmonary TB | Other Lung Disease | Healthy Subjects |
|---|---|---|---|---|---|
| Number | 70 | 18 | 36 | 45 | 76 |
| Age, mean yr ± SD | 68.0 ± 9.6 | 64.6 ± 11.6 | 52.9 ± 16.6* | 66.3 ± 10.9 | 38.1 ± 12.0* |
| Age range, yr | 50–90 | 28–78 | 24–76 | 29–82 | 20–65 |
| Sex, no. male/no. female | 25/45 | 10/8 | 26/10* | 34/11* | 41/35* |
| Duration of disease, mean yr ± SD | 4.8 ± 4.6 | | 0.3 ± 0.2 | 2.2 ± 2.4 | |

* $P < 0.001$.

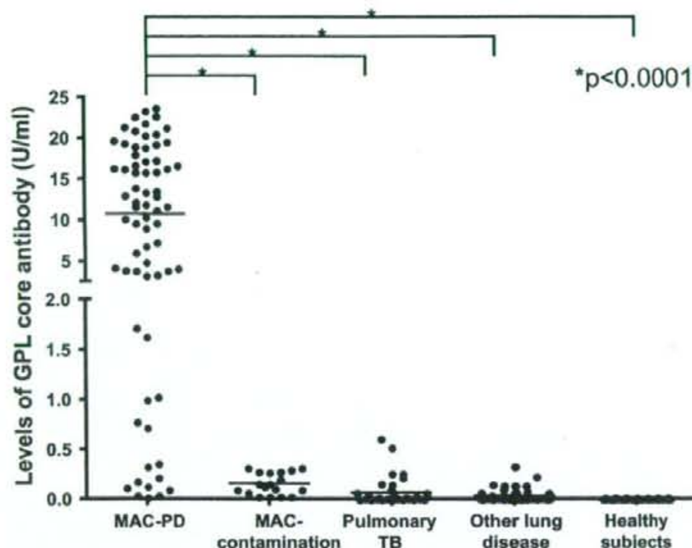


*Figure 1.* The level of serum IgA antibody to glycopeptidolipid (GPL) core antigen. Serum samples from six different institutions included 70 patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD), 18 with MAC contamination, 37 with pulmonary tuberculosis (TB), 45 with other lung diseases, and 76 healthy subjects. Antibody levels in MAC-PD were significantly higher than in the other groups ($P < 0.0001$). All results are expressed as individual data, and *horizontal bars* indicate geometric means.

levels of the antibody ($r = 0.43$, $P < 0.05$) (Figure 4). The total numbers of involved segments were not different (7.8 ± 4.9 and 7.9 ± 4.2 in fibrocavitary and NBE disease, respectively). Of 26 patients with NBE disease, 9 had small thin wall cavities. A tendency toward elevated GPL core antibody levels was found in NBE patients with cavities compared with those without, but this trend was not statistically significant ($P = 0.08$).

## DISCUSSION

We previously established a serologic test for MAC-PD using a mixture of GPLs and GPL core antigen, and reported the clinical application of the EIA method for quantifying antibody levels (7, 8). GPL is an antigen located on the surface of the MAC cell wall and determines the serotype. At present, 31 distinct serotype-specific GPLs have been identified, of which the complete structures of 14 have been identified (10–12). GPL consists of a core common to all MAC serotypes and a serotype-specific oligosaccharide. In the initial study to establish the serodiagnosis of MAC-PD, we used the whole GPL antigen, a mixture of 11 serotype-specific GPLs (7). We then found that the GPL core was the dominant antigenic epitope of GPL, and subsequently developed a serologic test using GPL core antigen (8). In the previous study, GPL core antibody (IgG, IgA, and IgM) levels were found to be elevated in sera of patients with MAC-PD, but not pulmonary TB, *M. kansasii*–PD, MAC colonization/contamination, and healthy subjects. The study showed that this serologic test was useful for diagnosing MAC-PD and for differentiating it from pulmonary TB and *M. kansasii*–PD. Consistent with this, Fujita and colleagues (13) reported elevated levels of antibody against the GPL core antigen in patients with MAC-PD but not in those with pulmonary TB. In our previous study (8), of the different Ig classes, best results were obtained by IgA, including an association with CCT findings. Thus, a higher level of serum IgA antibody to GPL core indicated a wider extent of MAC disease and larger nodule formation on CCT (9). Therefore, we have attempted to develop and to assess an EIA kit for quantifying serum IgA antibody to GPL core in the present study. Optical density levels were converted to U/ml using standard serum samples, which provided reliable and reproducible results. In this multicenter study, using the EIA kit, it was confirmed that patients with MAC-PD could be clearly differentiated from those with pulmonary TB, those with MAC contamination, those with other lung diseases, and healthy subjects. Similar to our previous studies (7–9), the sensitivity and specificity for diagnosing MAC-PD by the kit was high and the level of the antibody correlated with the extent of MAC-PD assessed using CCT.

Distinguishing pulmonary TB from MAC-PD in clinical practice using the EIA kit has proven useful. Differentiating TB from MAC is difficult because symptoms and radiographic findings are often similar among patients with pulmonary mycobacterial diseases. Patients with pulmonary TB require immediate treatment and isolation, whereas the diagnosis of MAC-PD does not necessitate rapidly starting antimicrobial therapy (1), and isolation is not required. GPL antigens, which are major cell surface antigens of MAC, are not present in the cell wall of *M. tuberculosis* complex (11). On the basis of this observation, patients with TB do not produce anti-GPL antibody. Indeed most patients with TB did not possess serum antibodies against GPLs (Figure 1) (7, 8). However, we cannot exclude the possibility that disease in patients with TB was of too short duration (MAC-PD, 4.8 ± 4.6 yr, vs. TB, 0.3 ± 0.2 yr) to have allowed immune responses and shed mycobacterial antigen. In this present study, with a cutoff level of 0.7 U/ml, all patients with TB were classified as seronegative. The levels of GPL core antibody in patients with pulmonary TB were very low or absent

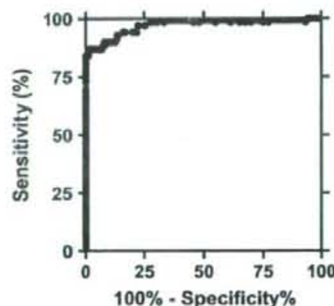


*Figure 2.* Receiver operating characteristic curve constructed for patients with *Mycobacterium avium*–complex pulmonary disease and the other groups.

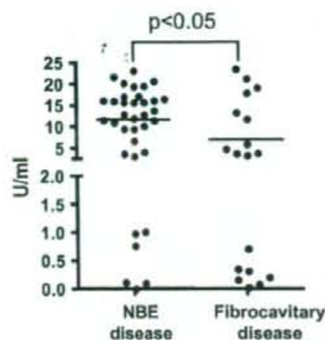


*Figure 3.* Levels of IgA antibody to glycopeptidolipid core antigens in nodular-bronchiectatic (NBE) and fibrocavitary subtypes of patients with *Mycobacterium avium* complex pulmonary disease (MAC-PD). Significantly higher levels were found in patients with MAC-PD with NBE compared with fibrocavitary disease ($P < 0.05$).

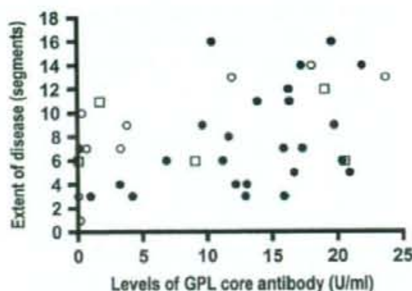


*Figure 4.* Correlation between antibody levels and radiographic severity using chest computed tomography in 41 patients with *Mycobacterium avium*–complex pulmonary disease. There was a positive correlation between the extent of disease and the levels of antibody ($r = 0.43$, $P < 0.05$). *Closed circles* represent patients with nodular-bronchiectatic disease, *open circles* represent patients with fibrocavitary disease, and *open squares* represent patients with unclassifiable type disease.

(0.1 ± 0.1 U/ml). In contrast, in previous studies (7, 8, 13), GPL seropositivity in patients with pulmonary TB ranged between 5.2 and 25%. One possible explanation for this previously reported lack of specificity may be that there was latent coinfection of MAC in patients with pulmonary TB. In the present study, however, we attempted to exclude patients with such latent coinfection because the entry criteria precluded patients having underlying lung disease or past history of pulmonary TB. Patients with lung diseases such as chronic obstructive pulmonary disease associated with smoking, bronchiectasis, previous mycobacterial disease, cystic fibrosis, and pneumoconiosis are prone to have MAC coinfection (1). In addition, future studies are needed to verify the cutoff value obtained from the ROC analysis using another sample of cases and controls on a much larger scale.

MAC-PD has recently been classified into two distinct subtypes: fibrocavitary disease and NBE disease (1). Fibrocavitary disease, the most common manifestation of MAC-PD, is usually seen in middle-aged or elderly men predisposed to lung disease due to smoking and alcohol drinking. This subtype of disease, generally progressive, is similar to pulmonary TB on chest radiography. If left untreated, it can lead to extensive lung destruction and death. In contrast, NBE disease is mostly seen in nonsmoking middle-aged or elderly women without predisposing lung disease. The clinical course is usually slower and less dramatic. Patients with NBE are presumed to have had a long subclinical period before appearance of disease manifestations. Significantly higher levels of GPL core antibody were seen in NBE than in fibrocavitary disease ($P < 0.05$) and higher seropositivity was found in patients with the former (91.4% compared with 63.2%). There were no significant differences of extent of disease between the two groups in patients who underwent CCT and serodiagnosis at the same time. Therefore, the results suggested the possibility that the antibody levels tend not to elevate in patients with fibrocavitary disease. This may reduce the utility of serodiagnosis for discriminating cavitary MAC from cavitary TB. However, the antibody would probably be present at high levels in patients with extensive lesions in fibrocavitary disease as was indeed found in three patients (17.9 ± 5.9 U/ml) who had extensive lesions (more than 13 segments) (Figure 4). Further investigations are required for confirmation of this notion in a larger study.

Of the 70 patients with MAC-PD, 64 had previously received combination chemotherapy, as recommended by the ATS guidelines (1). However, all had MAC-positive cultures at the time of serum collection, and were considered to have active MAC-PD. Thus, antibody levels were not changed by the failure of chemotherapy—that is, there was no conversion to seronegative from seropositive status (8); therefore, effects of the previous treatment on antibody levels were limited. Obviously, it would nonetheless be better to enroll chemotherapy-naive patients from diverse ethnic and racial populations and different geographic areas in future studies.

At present, the diagnosis of MAC-PD is usually made according to the ATS guidelines, which include clinical, radiographic, and microbiological criteria (1). The latter requires multiple positive cultures for MAC from sputum, a positive culture from bronchial lavage or a lung biopsy specimen, together with the other diagnostic features. Although it is easy to meet the criteria in advanced-stage MAC-PD, it is often difficult in early-stage disease. In clinical routine, it is impractical to obtain multiple sputum samples or perform bronchoscopy to obtain bronchial washings or lung tissue in all patients. It is also time consuming, because a long duration is required before the results of multiple cultures are available. There are several rapid methods for identification of MAC, but they have some limitations. The liquid culture–based system using radiometry and fluorometry allows the detection of mycobacterial growth at an early stage, fewer than 7 days for nontuberculous mycobacteria. However, limitations of this system include the inability to observe colony morphology, difficulty in recognizing mixed cultures, overgrowth by contaminations, cost, and radioisotope disposal. Rapid identification of MAC is also possible using DNA hybridization, nucleic acid amplification, or high-pressure liquid chromatography (1). The use of molecular biological technology has shortened the time required to identify mycobacteria from several weeks to as little as 1 day. The overall sensitivity for detecting MAC varies between 70 and 100%, with a specificity greater than 98%. However, the inability to distinguish live and dead organisms precludes nucleic acid amplification for definite diagnosis of active disease (14).

The EIA kit is a rapid (within a few hours) and noninvasive assay with high sensitivity (84.3%) and specificity (100%) for diagnosing MAC-PD. Using the EIA kit, as reported here, MAC-PD could be efficiently differentiated from MAC contamination. "MAC contamination" defined in the present study was considered to represent contamination from the environment, because patients were asymptomatic and revealed no significant CCT findings indicating active mycobacterial disease. Most of those people classified into the MAC contamination group were so categorized based on a single positive MAC culture by chance during the follow-up period after completion of chemotherapy for pulmonary TB or at routine examination on admission for other diseases. It is difficult to be certain that MAC contamina-

tion, as defined here, does not indicate subclinical infection because no confirmatory pathology was obtained. However, if MAC contamination does reflect subclinical infection, it is of little clinical importance and does not mandate therapy.

There were 15.7% false-negative EIA determinations in patients with MAC-PD. In such cases, diagnosis of MAC-PD should be made according to the ATS guidelines, as previously described. There are several possible explanations for these false-negative results, including the following: (*1*) recently diagnosed disease; (*2*) change of GPL core antigenicity after chemotherapy; or (*3*) diversity of immune responses to GPL core in individual patients, potentially governed by HLA genes (15). Therefore, it might be expected that not all patients with MAC-PD are capable of producing antibody to GPL core. Although the specificity determined here for the EIA kit was high, there remains also the possibility of false-positive results in patients with disease due to other mycobacteria, such as *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and *Mycobacterium scrofulaceum*, because these organisms also possess GPL on their cell wall surface (10, 11, 16). Indeed, we have detected seropositivity in several patients with culture-positive *M. fortuitum* (data not shown). The incidence of pulmonary disease due to these other mycobacteria is relatively low (<5%) in Japan and the United States (6, 17), but a report from South Korea documented a high incidence of pulmonary infection by *M. abscessus* or *M. fortuitum* (33 and 11%, respectively (18). Therefore, caution is necessary when interpreting the results of the EIA kit in locations where other mycobacterial infections are endemic.

A recent study using high-resolution CT documented that characteristic findings with multiple small nodular shadows combined with bronchiectasis are predictive for culture-positive MAC with a relatively high probability. Swenson and colleagues (19) reported that, of 15 patients with these characteristic findings, 8 (53%) had cultures positive for MAC. Tanaka and coworkers (20) reported that, of 26 similar patients, 13 (50%) had positive cultures for MAC in bronchial washings. Therefore, combining positive results obtained by the EIA and the characteristic findings of high-resolution CT should yield a definitive diagnosis of MAC-PD even in patients with sputum culture–negative results for MAC. This approach may be useful especially in elderly patients with complications, in whom bronchoscopy cannot be performed.

In summary, the EIA kit for detection of serum IgA antibody specific for GPL core antigen is useful for rapid and accurate serodiagnosis of MAC-PD. Taken together with clinical, radiographic, and microbiological criteria, the kit may be a valuable tool for the diagnosis of MAC-PD. Validation of the EIA kit in the diagnosis of MAC-PD requires a larger controlled study in diverse populations.

***Conflict of Interest Statement*****:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

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# Evaluation of antimicrobial susceptibility for β-lactams using the Etest method against clinical isolates from 100 medical centers in Japan (2006)

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**Abstract**

This antimicrobial resistance surveillance study was performed in 100 medical centers. Susceptibility testing (Etest; AB BIODISK, Solna, Sweden) of 9152 strains including *Escherichia coli* (991 strains), *Klebsiella* spp. (1000 strains), *Enterobacter* spp. (971 strains), *Citrobacter* spp. (803 strains), indole-positive *Proteae* spp. (834 strains), *Serratia* spp. (902 strains), *Acinetobacter* spp. (874 strains), *Pseudomonas aeruginosa* (992 strains), oxacillin-susceptible *Staphylococcus aureus* (984 strains), and coagulase-negative staphylococci (CoNS; 801 strains) was performed with 7 β-lactams (cefepime, cefpirome, ceftazidime, cefoperazone/sulbactam, imipenem and piperacillin for Gram-negative bacteria, or oxacillin for Gram-positive bacteria). No strain resistance to these β-lactams (except for ceftazidime) was found in oxacillin-susceptible *S. aureus* and CoNS. Of the *E. coli* clinical isolates, 17.1% were resistant to piperacillin, whereas 2.9% or less (cefpirome = 2.9%) were resistant to other β-lactam agents. *Klebsiella* spp. strains were more susceptible to imipenem (99.9%), cefepime (99.2%), ceftazidime (98.6%), and cefpirome (98.3%). Isolates of *Enterobacter* spp., *Citrobacter* spp., indole-positive *Proteae*, and *Serratia* spp. were susceptible to imipenem, cefepime, and cefpirome as well. *Acinetobacter* spp. strains were least resistant to cefoperazone/sulbactam (0.7% resistance), imipenem (2.6%), cefepime (6.6%), and ceftazidime (7.7%) compared with other β-lactam antibiotics tested. Isolates of *P. aeruginosa* were more susceptible to ceftazidime (8.7% resistance), cefoperazone/sulbactam (9.8%), and cefepime (8.9%) than piperacillin (11.9%), cefpirome (16.2%), and imipenem (12.4%). The percentage of imipenem-resistant *P. aeruginosa* was approximately 13% in clinical isolates in Japan. The proportion of strains resistant to β-lactam antimicrobials has been decreasing compared with data from 2004, suggesting that reduced consumption of β-lactams has reflected the decreased rates of resistant bacterial isolates in Japan.

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*Keywords:* β-Lactams; Etest; Susceptibility; Drug resistance

## 1. Introduction

β-Lactam antimicrobial agents have been widely used in clinical practice for more than 60 years. β-Lactamases have become the major resistance mechanism toward these agents in Gram-negative bacteria (Jacoby and Munoz-Price, 2005). Another prominent resistance mechanism in Gram-negative bacteria has been the decrease of antimicrobial concentrations to inhibit bacterial cell wall biosynthesis enzymes, for example, target enzymes (Alekshun and Levy, 2007).

Previously, extended-spectrum β-lactamase (ESBL)-producing organisms were reported in numerous countries worldwide (Canton and Coque, 2006). These ESBLs can hydrolyze penicillins and cephalosporins including oxyimino-cephalosporins. CTX-M–type ESBL enzymes prefer to hydrolyze cefotaxime as its major substrate (Ishii et al., 2007). These enzyme-producing isolates are found not only in clinical specimens (Canton and Coque, 2006; Ishii et al., 2005b) but also in animals and the environment in Japan (Ahmed et al., 2004; Kojima et al., 2005).

Plasmid-borne class B β-lactamases, metallo-β-lactamases (MBLs), are classified into 5 main molecular groups: IMP-, VIM-, SPM-, GIM-, and SIM-type enzymes (Walsh

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et al., 2005). These MBLs destroy most β-lactam antimicrobials including the carbapenems. IMP-1, the predominant MBL in Japan, has been found in clinical pathogens such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Acinetobacter baumannii*, *Achromobacter xylosoxidans*, *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter youngae*, *Klebsiella pneumoniae*, or *Shigella flexneri* (Walsh et al., 2005). MBL-producing *P. aeruginosa* and *Providencia rettgeri* isolates were detected in our previously reported surveillance program in 2002 (Ishii et al., 2002, 2005a, 2006; Kimura et al., 2005a; Shiroto et al., 2005). On the other hand, nosocomial infection caused by IMP-1–producing *K. pneumoniae* has occurred in general hospital in Japan (Fukigai et al., 2007). The isolation frequencies of MBL-producing *P. aeruginosa* in 2002 and 2004 were 1.9% and 2.3%, respectively (Ishii et al., 2005a, 2006).

A surveillance program by the Japan Antimicrobial Resistance Study Group was carried out from 1997 to 2004 (Ishii et al., 2002, 2005b, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). The present study was designed to provide up-to-date β-lactam antimicrobial susceptibility for clinical isolates including *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp. (*Proteus vulgaris*, *Providencia* spp., and *Morganella morganii*), *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, oxacillin-susceptible *Staphylococcus aureus* (MSSA), and oxacillin-susceptible coagulase-negative staphylococci (CoNS) in Japan. One hundred hospitals participated in this surveillance program during 2006. Participating centers represented all geographic regions in Japan. In the present study, we compare the incidences of β-lactam–resistant bacteria and the consumption of β-lactam antimicrobials in Japan.

## 2. Materials and methods

### 2.1. Bacterial isolates

The collection and subsequent testing of clinical isolates by the 100 participant centers began in July and was concluded in September 2006. Each participant center had an average of 632 beds. Fifty-five and 32 participating centers use MicroScan WalkAway system (Dade Behring, Tokyo, Japan) and Vitek system (bioMérieux, Tokyo, Japan) to identify the organisms, respectively. Twelve centers used other systems such as the BD Phoenix system (Becton Dickinson, Tokyo, Japan), Raisus system (Nissui Pharmaceutical, Tokyo, Japan) or API sires (bioMérieux), Enterotube system (Becton Dickinson), and so on. Each laboratory was instructed to construct a collection of consecutive bacterial strains of up to 10 nonduplicate patient isolates for each designated species groups (10 total) as stated in a prevalence format. These 10 organism groups were *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp., *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, MSSA (oxacillin MIC, ≤2 μg/mL), and oxacillin-susceptible CoNS (MIC, ≤0.25 μg/mL). The overall collection of bacterial strains from the 100 centers totaled 9152 strains including 991 *E. coli*, 1000 *Klebsiella* spp., 971 *Enterobacter* spp., 803 *Citrobacter* spp., 834 indole-positive *Proteus* spp., 902 *Serratia* spp., 874 *Acinetobacter* spp., 992 *P. aeruginosa*, 984 MSSA, and 801 oxacillin-susceptible CoNS.

The specimens from which the strains in this study were isolated are listed on Table 1. Although compliance was complete, 1 *S. aureus* strain was omitted from the analysis because the documented oxacillin-resistant criteria was redefined by the Clinical and Laboratory Standards Institute (CLSI, 2006) during the protocol period. Also, 6 proteae isolates were omitted from analysis because these strains were identified as *Proteus mirabilis*, an indole-negative proteae by the BD Phoenix system in the coordinating laboratory (Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Tokyo, Japan).

### 2.2. Antimicrobial susceptibility testing

Susceptibility testing of each isolate was determined by using Etest (AB BIODISK, Solna, Sweden) following the protocol described previously (Ishii et al., 2002, 2005a, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). Bacteria were cultured on a 90-mm-diameter Mueller–Hinton agar (Becton Dickinson) for 16 h at 35 °C. Isolated colonies were suspended in sterile saline to obtain a turbidity of 0.5 McFarland. Each cell suspension was spread on a 135-mm-diameter Mueller–Hinton agar plate (Becton Dickinson) with a cotton swab, and the Etest strips were placed on the plates according to the manufacturer's instructions. The following strips were used: oxacillin (for Gram-positive bacteria), piperacillin (for Gram-negative bacteria), ceftazidime, cefepime, cefpirome, cefoperazone/sulbactam, and imipenem. Results were recorded after 16 to 20 h of incubation at 35 °C except for *S. aureus* and CoNS for which incubation was extended to 24 h. MIC values were interpreted as the point of intersection of the inhibition ellipse with the Etest strips edge. All clinical laboratories used the same lot of Etest strips, Mueller–Hinton agar plates, and reference strains. Clinical and Laboratory Standards Institute (2007) does not have criteria (susceptible, intermediate, or resistant) for either cefpirome or cefoperazone/sulbactam. For comparison only, the same values for cefepime (CLSI, 2007) were used as criteria for cefpirome, and the value for cefoperazone alone were used as criteria for cefoperazone/sulbactam. All 100 hospitals provided their results to the Department of Microbiology and Infectious Diseases, Toho University School of Medicine, for analysis. If uncertain data were found in the provided results, including identification and susceptibility testing, all tests were repeated. Identification

Table 1
Specimens used in this study

| | *S. aureus* | CoNS | *E. coli* | *Klebsiella* spp. | *C. freundii* | *Enterobacter* spp. | Indole-positive *Proteae* | *Serratia* spp. | Acinetobacter spp. | *P. aeruginosa* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Urinary tract** | 57 | 108 | 547 | 252 | 274 | 157 | 375 | 148 | 75 | 220 |
| Urine | 27 | 65 | 373 | 156 | 182 | 95 | 233 | 92 | 42 | 116 |
| Urinary catheter | 12 | 10 | 117 | 74 | 71 | 40 | 123 | 51 | 23 | 88 |
| Others | 18 | 33 | 57 | 22 | 21 | 22 | 19 | 5 | 10 | 16 |
| **Pulmonary tract** | 392 | 96 | 106 | 411 | 112 | 418 | 85 | 487 | 542 | 449 |
| Sputum | 158 | 24 | 63 | 282 | 68 | 246 | 54 | 295 | 306 | 294 |
| BALF | 9 | 1 | 5 | 19 | 2 | 7 | | 6 | 9 | 5 |
| Intratracheal sputum | 26 | 1 | 16 | 42 | 11 | 55 | 20 | 105 | 92 | 84 |
| Pharyngeal mucus | 95 | 24 | 12 | 47 | 18 | 67 | 3 | 44 | 82 | 28 |
| Others | 104 | 46 | 10 | 21 | 13 | 43 | 8 | 37 | 53 | 38 |
| **Gastrointestinal tract** | 24 | 13 | 98 | 122 | 246 | 118 | 139 | 35 | 28 | 62 |
| Gastric or duodenal secretion | 0 | 2 | 6 | 16 | 9 | 12 | 5 | 5 | 7 | |
| Feces | 20 | 6 | 59 | 63 | 179 | 67 | 112 | 19 | 13 | 383 |
| Others | 4 | 5 | 33 | 43 | 58 | 39 | 22 | 11 | 8 | 21 |
| **Blood and fluids** | 83 | 223 | 104 | 95 | 33 | 77 | 23 | 56 | 68 | 81 |
| Blood | 55 | 182 | 87 | 75 | 24 | 50 | 14 | 42 | 57 | 27 |
| Spinal fluid | 1 | 7 | – | 1 | 1 | | | 1 | 3 | 1 |
| Others | 27 | 34 | 17 | 19 | 8 | 27 | 9 | 13 | 8 | 13 |
| **Others** | 428 | 361 | 136 | 120 | 138 | 201 | 212 | 176 | 161 | 220 |
| Drain fluid (thoracic cavity abdominal cavity) | 15 | 10 | 26 | 24 | 25 | 37 | 32 | 19 | 18 | 29 |
| Ophthalmic secretion | 29 | 57 | 1 | 1 | 3 | 1 | 7 | 8 | 7 | 6 |
| Ear secretion | 95 | 53 | 3 | 7 | 4 | 23 | 10 | 20 | 23 | 36 |
| Abscess | 167 | 98 | 71 | 57 | 72 | 69 | 94 | 73 | 57 | 90 |
| Skin or decubitus | 83 | 55 | 14 | 9 | 17 | 17 | 50 | 26 | 23 | 27 |
| **Unspecified** | 39 | 88 | 21 | 22 | 17 | 54 | 19 | 30 | 33 | 32 |

BALF = bronchoalveolar lavage fluid.

and determination of MIC values was performed using the BD Phoenix system.

### 2.3. Quality control

For quality control (QC) of the Etest strips, the following reference strains were used: *E. coli* ATCC25922, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 (CLSI, 2006). CLSI does not have MIC QC ranges regarding cefpirome and cefoperazone/sulbactam. In this study, a range was determined near (±1 doubling dilution) the median MIC for cefepime and cefoperazone/sulbactam, as what was done in our previous reports (Ishii et al., 2006). The laboratories were required to test a set of all QC organisms in a replicate manner.

## 3. Results

### 3.1. Quality assurance

The validity of generated data was assured by employing appropriate QC and quality assurance measures. Values obtained for the challenge set of strains resulted in 28 of 1073 values falling out of the appropriate susceptibility category (2.6%). Of these 2.6%, 0.2% ($n = 2$ strains) were very major (false-susceptible) errors and 0.4% ($n = 4$ strains) were major (false-resistant) errors. Overall, this equates to 97.4% of MIC categoric results being accurate.

### 3.2. Activity against staphylococci

Because the CLSI (2006, 2007) recommends that oxacillin-resistant staphylococci be considered as resistant to all β-lactams, only oxacillin-susceptible strains were collected in this study. Of all the tested staphylococci, 984 isolates of *S. aureus* and 801 isolates of oxacillin-susceptible CoNS strains were susceptible to cefepime, cefpirome, cefoperazone/sulbactam, and imipenem (Table 2). However, 22 *S. aureus* (0.7%) and 23 oxacillin-susceptible CoNS (1.1%) were resistant to ceftazidime. The rank order of activity for all the tested agents using $MIC_{90}$ values was imipenem (0.032 μg/mL) > oxacillin > cefpirome > cefoperazone/sulbactam > cefepime > ceftazidime (12–16 μg/mL).

### 3.3. Activity against E. coli and Klebsiella spp.

A total of 991 *E. coli* and 1000 *Klebsiella* spp. isolates were tested. Generally, all agents tested, except piperacillin (17.1% resistant), were highly active against *E. coli* and *Klebsiella* spp. (Table 2). No imipenem-resistant strains of *E. coli* or *Klebsiella* spp. were observed in this study.

### 3.4. Activity against other Enterobacteriaceae

*Enterobacter* spp. and *Citrobacter freundii* showed lower rates of susceptibility to piperacillin (77.6–83.4%), ceftazidime (79.5–83.0%), and cefoperazone/sulbactam

Table 2
Antimicrobial activity of 7 tested β-lactams against clinical isolates (2006)

| Organism (no. tested) | Antimicrobial agent | MIC (μg/mL) | | MIC (μg/mL) | Category (%) | |
|---|---|---|---|---|---|---|
| | | 50% | 90% | Range | S | R |
| *S. aureus* (984) | Oxacillin | 0.38 | 0.5 | 0.023–2 | 100.0 | 0.0 |
| | Ceftazidime | 12 | 16 | 0.125–48 | 24.7 | 0.7 |
| | Cefepime | 3 | 4 | 0.032–8 | 100.0 | 0.0 |
| | Cefpirome | 1 | 1.5 | 0.032–4 | 100.0 | 0.0 |
| | CP/SB | 2 | 3 | 0.032–6 | 100.0 | 0.0 |
| | Imipenem | 0.032 | 0.032 | <0.016–2 | 100.0 | 0.0 |
| Coagulase-negative staphylococci (801) | Oxacillin | 0.19 | 0.25 | <0.016–0.25 | 100.0 | 0.0 |
| | Ceftazidime | 6 | 12 | 0.125–>256 | 85.5 | 1.1 |
| | Cefepime | 1 | 2 | 0.064–>256 | 99.8 | 0.2 |
| | Cefpirome | 0.38 | 0.75 | 0.023–>256 | 99.6 | 0.2 |
| | CP/SB | 1 | 2 | 0.064–>256 | 99.6 | 0.2 |
| | Imipenem | 0.023 | 0.032 | <0.016–>256 | 99.8 | 0.2 |
| *E. coli* (991) | Piperacillin | 2 | >256 | 0.023–>256 | 72.9 | 17.1 |
| | Ceftazidime | 0.125 | 0.75 | 0.023–>256 | 97.2 | 2.4 |
| | Cefepime | 0.032 | 0.125 | <0.016–>256 | 97.4 | 1.4 |
| | Cefpirome | 0.047 | 0.125 | <0.016–>256 | 95.5 | 2.9 |
| | CP/SB | 0.25 | 2 | <0.016–>256 | 98.7 | 0.4 |
| | Imipenem | 0.25 | 0.38 | <0.016–4 | 100.0 | 0.0 |
| *Klebsiella* spp. (1000) | Piperacillin | 6 | 48 | 0.125–>256 | 86.5 | 8.5 |
| | Ceftazidime | 0.125 | 0.5 | <0.016–>256 | 98.6 | 1.0 |
| | Cefepime | 0.047 | 0.125 | <0.016–96 | 99.2 | 0.4 |
| | Cefpirome | 0.047 | 0.125 | <0.016–>256 | 98.3 | 1.0 |
| | CP/SB | 0.25 | 2 | <0.016–>256 | 96.4 | 3.0 |
| | Imipenem | 0.25 | 0.38 | 0.023–6 | 99.9 | 0.0 |
| *C. freundii* (803) | Piperacillin | 2 | >256 | 0.032–>256 | 77.6 | 17.9 |
| | Ceftazidime | 0.5 | >256 | 0.047–>256 | 79.5 | 18.2 |
| | Cefepime | 0.032 | 1.5 | <0.016–>256 | 98.9 | 0.6 |
| | Cefpirome | 0.064 | 3 | <0.016–>256 | 96.1 | 2.2 |
| | CP/SB | 0.5 | 16 | 0.023–>256 | 90.4 | 2.9 |
| | Imipenem | 0.5 | 1 | 0.032–4 | 100.0 | 0.0 |
| *Enterobacter* spp. (971) | Piperacillin | 2 | 128 | 0.047–>256 | 83.4 | 10.5 |
| | Ceftazidime | 0.25 | 96 | 0.023–>256 | 83.0 | 13.7 |
| | Cefepime | 0.047 | 1 | <0.016–>256 | 98.4 | 0.6 |
| | Cefpirome | 0.064 | 2 | <0.016–>256 | 96.5 | 1.5 |
| | CP/SB | 0.38 | 16 | <0.016–>256 | 91.6 | 3.5 |
| | Imipenem | 0.5 | 1 | <0.064–12 | 99.4 | 0.0 |
| Indole-positive *Proteae* (834) | Piperacillin | 0.5 | 6 | 0.047–>256 | 92.9 | 4.9 |
| | Ceftazidime | 0.094 | 1 | 0.016–>256 | 95.4 | 2.6 |
| | Cefepime | 0.032 | 0.125 | <0.016–>256 | 99.4 | 0.2 |
| | Cefpirome | 0.064 | 0.38 | <0.016–>256 | 98.6 | 0.8 |
| | CP/SB | 1 | 3 | 0.032–>256 | 98.9 | 0.5 |
| | Imipenem | 1.5 | 3 | 0.047–>256 | 98.3 | 0.6 |
| *Serratia* spp. (902) | Piperacillin | 2 | 48 | 0.064–>256 | 83.3 | 6.9 |
| | Ceftazidime | 0.19 | 1 | 0.023–>256 | 96.7 | 2.5 |
| | Cefepime | 0.064 | 0.75 | <0.016–>256 | 97.9 | 0.9 |
| | Cefpirome | 0.064 | 0.5 | <0.016–>256 | 97.8 | 1.2 |
| | CP/SB | 1 | 16 | 0.032–>256 | 92.7 | 4.9 |
| | Imipenem | 0.5 | 1 | <0.016–>256 | 99.4 | 0.6 |
| *Acinetobacter* spp. (874) | Piperacillin | 12 | 64 | 0.032–>256 | 91.8 | 7.8 |
| | Ceftazidime | 4 | 16 | 0.032–>256 | 88.1 | 7.7 |
| | Cefepime | 2 | 12 | 0.023–>256 | 88.2 | 6.6 |
| | Cefpirome | 2 | 16 | 0.023–>256 | 88.9 | 8.5 |
| | CP/SB | 2 | 4 | 0.125–>256 | 97.6 | 0.7 |
| | Imipenem | 0.38 | 1 | 0.032–>256 | 95.3 | 2.6 |
| *P. aeruginosa* (992) | Piperacillin | 4 | >256 | 0.25–>256 | 87.4 | 11.9 |
| | Ceftazidime | 2 | 16 | 0.19–>256 | 87.0 | 8.7 |
| | Cefepime | 3 | 24 | 0.19–>256 | 79.3 | 8.9 |
| | Cefpirome | 4 | 64 | 0.125–>256 | 70.7 | 16.2 |
| | CP/SB | 4 | 48 | 0.25–>256 | 80.5 | 9.8 |
| | Imipenem | 1.5 | 16 | 0.094–>256 | 74.8 | 12.4 |

CP/SB = cefoperazone/sulbactam (2:1); S = susceptible; R = resistance.

(90.4–91.6%) compared with the other tested β-lactams (Table 2). Susceptibility rates for cefepime (98.4–98.9%) and imipenem (99.4–100%) were superior to cefpirome (96.1–96.5%). For the indole-positive *Proteus* spp., susceptibility rates of piperacillin (92.9%) and ceftazidime (95.4%) were lower than for the other β-lactam antibiotics. *Serratia* spp. also showed lower rates of susceptibility to piperacillin (83.3%) and cefoperazone/sulbactam (92.7%) compared with the other tested β-lactams (96.7–99.4%).

### 3.5. Activity against nonfermentative Gram-negative bacilli

For *Acinetobacter* spp., cefoperazone/sulbactam was the most active antimicrobial (combination) (97.6% susceptible), followed by imipenem (95.3%), cefpirome (88.9%), cefepime (88.2%), and ceftazidime (88.1%). Piperacillin (91.8%) showed the lowest susceptibility rate when compared with the other tested β-lactams (Table 2).

## 4. Discussion

All centers participating in this surveillance were not small-sized hospitals (average number of beds = 632), so results reflect large hospital data. Imipenem maintained antimicrobial activity against Gram-positive and Gram-negative bacteria except for some indole-positive *Proteus* spp., *Acinetobacter* spp., and *P. aeruginosa* (Table 2) compared with previous studies (Ishii et al., 2002, 2005a, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). Against *Acinetobacter* spp., the combination of cefoperazone and sulbactam had the most potent antimicrobial effect. Sulbactam has been recognized as one of the effective agents against carbapenem-resistant *A. baumannii* (Go et al., 1994). In 2004, the resistance rates of *Acinetobacter* spp. to cefepime or cefpirome were 7.6% and 11.6%, respectively (Ishii et al., 2006). The present surveillance data show that the resistance rates for cefepime and cefpirome were only 7.0% and 8.6%, respectively. Multidrug-resistant (MDR) *Acinetobacter* spp. isolates have become a problem in Europe (Paterson, 2006). Fortunately, these reported data suggested that expanded-spectrum cephalosporin-resistant *Acinetobacter* spp. are not increasing in Japan.

ESBL-producing Enterobacteriaceae are well known as cephalosporin-resistant strains (Ahmed et al., 2004). In this study, 6.2% (61 strains) of *E. coli* and 4.2% (42 strains) of *K. pneumoniae* showed a MIC value of 2 μg/mL or more for ceftazidime, which suggests that they are ESBL producers according to the CLSI (2006). Among *Klebsiella* spp., 26 *K. pneumoniae* and 16 *Klebsiella oxytoca* isolates were possible ESBL producers according to this screening test. These *Klebsiella* spp. isolates were collected in 33 hospitals, and the *E. coli* were isolated from 40 hospitals. ESBL producers were confirmed by the CLSI disk with clavulanate test (2007). Because some *K. oxytoca* strains produce K1 enzyme, which behaves like an ESBL and in which it was impossible to separate the K1 β-lactamase from an ESBL by a phenotypic

Table 3
Annual changing of resistant percentages of 7 β-lactams against clinical isolates (1997–2006)

| Organism | Antimicrobial agents | Year (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1997 | 1998 | 2000 | 2002 | 2004 | 2006 |
| *S. aureus* | Oxacillin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Ceftazidime | 6.4 | 0.5 | 2 | 2.4 | 2.2 | 0.7 |
| | Cefepime | 0 | 0 | 0 | 0 | 0 | 0 |
| | Cefpirome | 0 | 0.5 | 0 | 0 | 0 | 0 |
| | CP–SB | 0 | 0 | 0 | 0 | 0 | 0 |
| | Imipenem | 0 | 0 | 0 | 0 | 0 | 0 |
| CoNS | Oxacillin | 0 | 0 | 0 | 0 | 0 | 0 |
| | Ceftazidime | 15.4 | 1.6 | 2.1 | 3.9 | 2.9 | 1.1 |
| | Cefepime | 0 | 0 | 0 | 0 | 0.7 | 0.2 |
| | Cefpirome | 0 | 0 | 0 | 0 | 0.6 | 0.2 |
| | CP–SB | 0 | 0 | 0 | 0 | 0.5 | 0.2 |
| | Imipenem | 0 | 0 | 0 | 0 | 0.7 | 0.2 |
| *E. coli* | Piperacillin | 14.6 | 12.6 | 11.9 | 10.8 | 16.5 | 17.1 |
| | Ceftazidime | 0.5 | 0 | 1 | 0.5 | 1 | 2.4 |
| | Cefepime | 0.5 | 0 | 0.5 | 0.7 | 0.9 | 1.4 |
| | Cefpirome | 0.5 | 0 | 1 | 1.3 | 1.5 | 2.9 |
| | CP–SB | 0.5 | 0 | 0.5 | 1 | 0.7 | 0.4 |
| | Imipenem | 0.5 | 0 | 0 | 0 | 0 | 0 |
| *Klebsiella* spp. | Piperacillin | 7.2 | 9.6 | 7.2 | 7.4 | 11.2 | 8.5 |
| | Ceftazidime | 1.8 | 0.9 | 0.2 | 1 | 1.1 | 1 |
| | Cefepime | 0 | 0 | 0.2 | 0.2 | 0.8 | 0.4 |
| | Cefpirome | 1.4 | 0.5 | 0.7 | 0.3 | 1.4 | 1 |
| | CP–SB | 2.7 | 1.4 | 1.5 | 2.5 | 3.9 | 3 |
| | Imipenem | 0 | 0 | 0 | 0 | 0.2 | 0 |
| *C. freundii* | Piperacillin | 26.1 | 22.6 | 18.4 | 18.7 | 19.2 | 17.9 |
| | Ceftazidime | 25 | 22.1 | 19.5 | 19.7 | 16.7 | 18.2 |
| | Cefepime | 0 | 0 | 0.6 | 0.6 | 1.6 | 0.6 |
| | Cefpirome | 1.1 | 2.1 | 1.7 | 1.6 | 2 | 2.2 |
| | CP–SB | 8.3 | 6.3 | 5.8 | 2 | 5.9 | 2.9 |
| | Imipenem | 0 | 0 | 0 | 0.2 | 0.1 | 0 |
| *Enterobacter* spp. | Piperacillin | 18.5 | 25.1 | 18 | 15 | 14.5 | 10.5 |
| | Ceftazidime | 20.5 | 24.2 | 22.8 | 20.2 | 16.8 | 13.7 |
| | Cefepime | 1 | 0.5 | 1.3 | 2.1 | 1.7 | 0.6 |
| | Cefpirome | 3.9 | 4.3 | 5.3 | 3.7 | 3.4 | 1.5 |
| | CP–SB | 15.1 | 10.6 | 8.5 | 5.9 | 7.1 | 3.5 |
| | Imipenem | 0.5 | 0.5 | 0 | 0.5 | 0.1 | 0 |
| Indole-positive *Proteae* | Piperacillin | 8.7 | 8 | 6.3 | 5.5 | 6 | 4.9 |
| | Ceftazidime | 0.5 | 3 | 2.6 | 4.5 | 2 | 2.6 |
| | Cefepime | 0.5 | 0.5 | 0 | 1.4 | 1.2 | 0.2 |
| | Cefpirome | 3.1 | 0.5 | 0 | 0.6 | 1.9 | 0.8 |
| | CP–SB | 1.5 | 0 | 1.7 | 2.4 | 0.7 | 0.5 |
| | Imipenem | 1 | 5 | 0.9 | 3.3 | 0.7 | 0.6 |
| *Serratia* spp. | Piperacillin | 25 | 22.3 | 15 | 9.8 | 10.1 | 6.9 |
| | Ceftazidime | 9.5 | 6.8 | 8 | 7.1 | 3.7 | 2.5 |
| | Cefepime | 5 | 5.8 | 6.5 | 5.3 | 3.2 | 0.9 |
| | Cefpirome | 8.5 | 6.3 | 7.8 | 4.7 | 3.2 | 1.2 |
| | CP–SB | 23.5 | 16 | 14.2 | 10.9 | 6.1 | 4.9 |
| | Imipenem | 4.5 | 4.4 | 4.5 | 3.6 | 1.5 | 0.6 |
| *Acinetobacter* spp. | Piperacillin | 31.2 | 30.2 | 5.9 | 9.3 | 13.3 | 7.8 |
| | Ceftazidime | 8 | 4 | 4.5 | 5.8 | 6 | 7.7 |
| | Cefepime | 5 | 8 | 5.1 | 7.6 | 7 | 6.6 |
| | Cefpirome | 12.1 | 15 | 5.4 | 11.6 | 8.6 | 8.5 |
| | CP–SB | 0.5 | 0.5 | 0.3 | 1.5 | 0.8 | 0.7 |
| | Imipenem | 2.5 | 6.5 | 3.1 | 5 | 3.2 | 2.6 |
| *P. aeruginosa* | Piperacillin | 20.1 | 18.5 | 15.7 | 15 | 15.5 | 11.9 |
| | Ceftazidime | 11.4 | 8.7 | 10.8 | 12.3 | 9.9 | 8.7 |
| | Cefepime | 9.1 | 9.1 | 12.5 | 12.6 | 11.2 | 8.9 |
| | Cefpirome | 27.9 | 27.2 | 26 | 22.6 | 19.1 | 16.2 |
| | CP–SB | 13.7 | 11.5 | 13.2 | 12.5 | 14.9 | 9.8 |
| | Imipenem | 22.4 | 24.9 | 20.3 | 30.8 | 19.3 | 12.4 |

CP–SB = cefoperazone/sulbactam (2:1).

test, all *K. oxytoca* isolates were omitted from the confirmatory testing. Thirteen *E. coli* strains (1.3%) and 4 *K. pneumoniae* strains (0.4%) were confirmed as ESBL producers. These values are the same as our previous report. Moreover, 2 *C. freundii* isolates (0.2%) were confirmed as ESBL producers by the CLSI disk with clavulanate test using Mueller–Hinton agar plates in the presence of 300 μg/mL of 3-aminophenyl boronic acid (final concentration) as a specific inhibitor of class C β-lactamases (Yagi et al., 2005). This result suggested that it is important to survey ESBL producers, not only *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis*, but also the other Enterobacteriaceae.

Twenty-five strains of *P. aeruginosa* (2.5%) were confirmed as MBL producers in this surveillance program by using imipenem and ceftazidime disk in the presence/absence of dipicolinic acid (Kimura et al., 2005b). Of the *P. aeruginosa*, 1.9% and 2.3% produced MBL in 2002 and 2004, respectively (Ishii et al., 2005a and 2006). The present data suggest that MBL-producing *P. aeruginosa* are increasing in Japan. On the other hand, imipenem-resistant *P. aeruginosa* were present in 18.6% (185 isolates) of the isolates in this study. So, this result suggests that class B β-lactamases are not the main mechanism for carbapenem resistance in *P. aeruginosa*. In 2006, MBL producers among the tested Enterobacteriaceae and *Acinetobacter* spp. were present in 0.2% (13 isolates) and 0.2% (2 isolates) by phenotypic testing. The isolation frequency of Enterobacteriaceae producing an MBL (2004) has the same value (0.2%: 12/5596 isolates). On the other hand, MBL producers of *Acinetobacter* spp. decreased from 1.2% (2004) to 0.2%. In 2004, MBL-producing *Acinetobacter* spp. (11 isolates) were isolated from only 5 hospitals.

MDR *P. aeruginosa* is a serious problem in the world (Paterson, 2006). These MDR organisms are resistant to carbapenems, fluoroquinolones, and aminoglycosides. We determined additional antimicrobial susceptibility testing results for imipenem-resistant *P. aeruginosa* by using the BD Phoenix system. Nineteen isolates showed resistance to amikacin and 119 isolates to levofloxacin (data not shown). Eighteen amikacin-resistant isolates were also resistant to levofloxacin, so the incidence of MDR *P. aeruginosa* was 1.7% (17 strains). This present data suggest, however, that the isolation frequency of MDR *P. aeruginosa* was not increasing compared with the 2004 results.

All tested Gram-negative organisms with the exception of *E. coli* and *Acinetobacter* spp. improved their susceptibilities for β-lactams (Table 3). For example, the percentages of imipenem-resistant isolates of *Serratia* spp. were 4.5% (1997), 4.4% (1998), 4.5% (2000), 3.6% (2002), 1.5% (2004), and 0.6% (2006) (Ishii et al., 2002, 2005a, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). Fig. 1 illustrates the consumption of β-lactams in Japan from 1997 to 2006 (IMS-Japan K.K., Tokyo, Japan, agreed to present this data). These data indicate that the consumption of β-lactam exception of penicillins has been decreasing year by year. Also, these results suggest that regulation of antimicrobial usage and dosing can improve antimicrobial susceptibility patterns for Japanese clinical isolates.

In conclusion, the susceptibility of *P. aeruginosa* to almost β-lactam antimicrobial agents has improved compared with previous reported years. Overall, cefepime is maintaining its in vitro activity against Gram-positive and Gram-negative bacteria. It is very important to continue

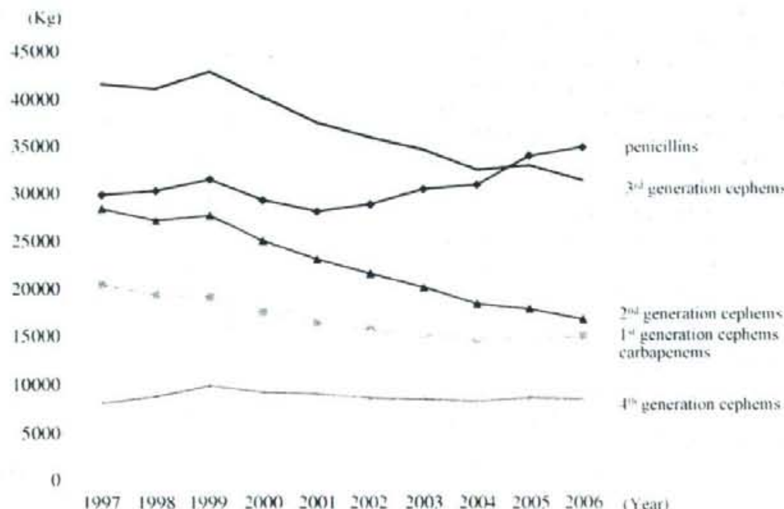


Fig. 1. The consumption of β-lactam antibiotics in Japan during 1997 to 2006 (Copyright 2007 IMS Japan. All rights reserved. Source: IMS JPM. Reprinted with permission).

surveillance for the MDR bacteria because of limited treatment options that could lead to optimal outcomes.

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# $CD4^+$ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin

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BCG; urease; macrophage; dendritic cell.

## Abstract

We constructed a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-ΔUT) that lacks urease, providing acidic intraphagosomal conditions to drive an effective human immune T-cell response. BCG-ΔUT-infected macrophages stimulated autologous $CD4^+$ T cells more efficiently than parent BCG-infected macrophages. For further T-cell activation, BCG-ΔUT-infected macrophages required pretreatment with exogenous recombinant granulocyte-macrophage colony-stimulating factor or costimulation with either CD40 ligand or interferon-γ. By contrast, BCG-ΔUT-infected dendritic cells induced significant activation of naïve $CD4^+$ T cells without costimulating signals. C57BL/6 mice intradermally inoculated with BCG-ΔUT more efficiently produced memory T cells that responded to recall antigen. Therefore, the depletion of urease from BCG is useful for the activation of T cells.

## Introduction

Mycobacteria, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, are representative parasitic intracellular pathogens. *Mycobacterium leprae* is a causative agent of human leprosy, in cases of which skin lesions and chronic progressive peripheral nerve injury are usually observed (Stoner, 1979; Job, 1989). At present, around one-third of individuals are infected with *M. tuberculosis* and several millions die as result of tuberculosis each year (Dye *et al.*, 2005; World Health Organization, 2006). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been used as a vaccine against leprosy, although its efficacy is quite limited (Andersen & Doherty, 2005; Setia *et al.*, 2006). The emergence of multidrug-resistant strains of these mycobacteria is of concern (Maeda *et al.*, 2001; Kai *et al.*, 2004; Kaufmann, 2005), and therefore the urgent development of a new vaccine, including a more efficacious recombinant BCG, is desired (Kaufmann, 2005).

Among various immunocompetent cells, $CD4^+$ T cells, especially IFN-γ-producing cells, play an extremely important role in inhibiting the multiplication of mycobacteria, killing them in the early stages of infection, and keeping the bacterial load at a stable level (Orme *et al.*, 1993; Dockrell *et al.*, 1996; Hashimoto *et al.*, 2002). $CD4^+$ T cells that can respond quickly to pathogenic mycobacteria and produce IFN-γ are known as memory T cells. The efficient production of such memory T cells needs pre-exposure to antigenic vaccinating molecules, which share their antigenicity with that of pathogenic mycobacteria (Kaufmann, 2006). BCG has been considered a good candidate for a vaccine against *M. leprae* in this respect, however its efficacy is limited in several aspects, including the ability to activate T cells (Kaufmann & McMichael, 2005). BCG resides in the phagosomes of macrophages and thus attenuates the trafficking of antigenic molecules to the macrophage cell surface (Grode *et al.*, 2005). One possible strategy for improving the ability of BCG to stimulate T cells is to enhance its ability to fuse with the lysosomes. To this end, we knocked out the *urease* gene from BCG. The urease-deficient recombinant BCG (BCG-ΔUT) is expected to allow phagosomal acidification in the host cells, and induce efficient phagosome maturation for cytolytic activity of the antigenic molecules of BCG (Schaible *et al.*, 1998; Honerzu Bentrup & Russell, 2001).

In the present study, we evaluated the ability of BCG-ΔUT to activate IFN-γ-producing type 1 $CD4^+$ T cells through

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antigen-presenting cells (APCs), and to produce memory $CD4^+$ T cells. When used as a target of BCG-ΔUT, macrophages fully stimulated $CD4^+$ T cells in the presence of costimulatory agents such as CD40 ligand (L) and IFN-γ. In addition, BCG-ΔUT-infected monocyte-derived dendritic cells (DCs) activated type 1 $CD4^+$ T cells more efficiently than parent BCG-infected cells in the absence of these costimulators. Therefore, BCG-ΔUT was found to be a useful T-cell-stimulating agent.

## Materials and methods

### Preparation of blood cells

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. PPD-negative individuals provide more information, however, as healthy individuals are PPD-positive, due to compulsory BCG vaccination for children in Japan (0–4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% fetal calf serum and recombinant (r) macrophage colony-stimulating factor (M-CSF) (R&D Systems, Abingdom, UK) (Makino *et al.*, 2007). Macrophages were pulsed with rBCGs on day 5 of culture, and were used as a stimulator of T cells on day 7 (Makino *et al.*, 2007). Monocyte-derived DCs were differentiated as described previously (Makino *et al.*, 1999). Briefly, monocytes were cultured in the presence of 50 ng recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Pepro Tech EC Ltd, London, UK) and 10 ng of recombinant interleukin (rIL)-4 (Pepro Tech) per millilitre (Makino *et al.*, 1999). On day 3 of culture, immature DCs were infected with rBCGs at the indicated multiplicity of infection (MOI), and on day 5 of culture, DCs were used for further analyses of surface antigens and for mixed-lymphocyte assays.

### BCG culture and DNA manipulation

The mycobacterial strain, BCG substrain Tokyo, for DNA manipulation was grown in Middlebrook 7H9 broth (Difco Laboratories) with 0.05% Tween 80 or Middlebrook 7H10 agar (Difco) with 0.5% glycerol, each supplemented with 10% albumin–dextrose–catalase enrichment (Difco). DNA manipulations including isolation of DNA, transformation and PCR, were carried out as described previously (Miyamoto *et al.*, 2004). *Escherichia coli* strain DH5α was used for routine manipulation and the propagation of plasmid DNA. *Escherichia coli* strain STBL4 was used for the construction of plasmid vectors derived from phAE87. Antibiotics were added as required: hygromycin B, 150 $\mu g\,mL^{-1}$ for *E. coli* and 75 $\mu g\,mL^{-1}$ for *Mycobacterium smegmatis* ($mc^2$155) and *M. bovis* BCG. A recombinant BCG that lacks a *urease* gene was constructed. The sequence of the targeted gene, *ureC* (BCG 1886), was obtained from the BCG list (http://genolist.pasteur.fr/BCGList/). The *ureC* gene was inactivated by inserting a hygromycin-resistance cassette (*hyg*) using a specialized transducing phage system for homologous recombination (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). To construct the disrupted sequence, fragments of around 0.9 kb both upstream and downstream of *ureC* were amplified from BCG-Tokyo genomic DNA using the following two pairs of primers: F UureC and R UureC for upstream of *ureC*, and F DureC and R DureC for downstream of *ureC*. The PCR products were digested with each restriction enzyme and cloned into the corresponding site flanking *hyg* of pYUB854 to give pYUB854-ureC-UD. This plasmid was used for packaging into the phasmid vector phAE87 to construct a specialized transducing mycobacteriophage for gene disruption as described previously (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). BCG-Tokyo infected with the mycobacteriophage at an MOI of 50 was incubated at 37 °C for 3 h in 7H9 broth without Tween 80. Harvested bacterial cells were then plated and cultured on 7H10 agar containing hygromycin B (75 $\mu g\,mL^{-1}$) for 3 weeks. The hygromycin B-resistant colonies were selected and evaluated with a conventional urease assay. A change in the color of the assay medium from yellowish to red was scored as urease-positive. Furthermore, genomic DNA obtained from these colonies was subjected to PCR to confirm the disruption of the gene using primers F ureC and R ureC (Fig. 1). The colony which tested negative in the urease assay was named BCG-ΔUT, while the parental BCG substrain Tokyo is referred to as BCG-Tokyo.

### Preparation of *M. leprae*

*Mycobacterium leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted by Shepard's method (Charles & Shepard, 1960). The MOI for infection to host cells was determined based on the assumption that macrophages and DCs were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002).



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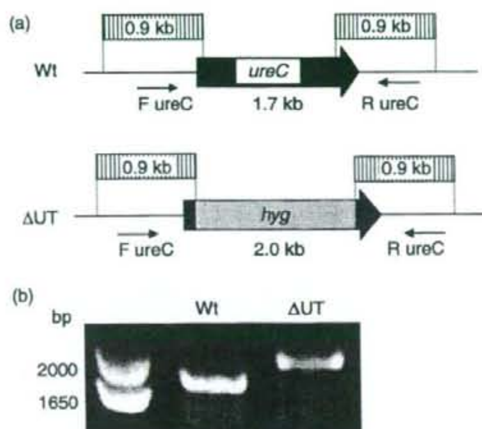


**Fig. 1.** Disruption of the *ureC* gene. (a) Schematic diagram of the *ureC* region on the chromosome of the wild-type *Mycobacterium bovis* BCG Tokyo strain and its gene disruptant, ΔUT. The shaded boxes indicate the regions included in the recombinant phage for gene disruption. The black arrow represents the coding region of the *ureC* gene. The gray box represents the hygromycin-resistace cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (b) PCR analysis of the wild-type and the disruptant using the primers indicated above.

### Preparation of mycobacterial antigen

The cytosolic fraction of BCG-Tokyo (BCC) was obtained as described previously (Maeda *et al.*, 2003). Briefly, the mycobacterial suspension containing the protease inhibitors was mixed with zirconium beads at a ratio of *c.* 1 : 1 (v/v) and homogenized using a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo). The suspension was centrifuged at 10 000 *g* to remove the cell-wall fractions. The supernatant was then ultracentrifuged at 100 000 *g* and the resulting supernatant was taken as the cytosolic fraction. For preparation of the *M. leprae* membrane (MLM) fraction, *M. leprae* was used instead of BCG and treated similarly. The pellet obtained by ultracentrifugation (100 000 *g* for 1 h) was used as a membrane fraction (MLM). The optimal concentration of BCC and MLM for stimulating T cells was determined in advance.

### Analysis of cell surface antigens

The expression of cell surface antigens on macrophages and DCs, either untreated or treated with exogenous rIFN-γ (R&D Systems), was analyzed using a FACSCalibur flow cytometer. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and $1 \times 10^4$ live cells were analyzed. For the analysis of cell surface antigens, the following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated mAbs against HLA-ABC (G46-2.6), HLA-DR (L243), CD14 (M5E2), CD40 (5C3) and CD86 (FUN-1). These mAbs were obtained from BD PharMingen (San Diego, CA).

### APC function of rBCG-infected macrophages and DCs

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous mixed-lymphocyte assay as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMCs by using a CD4-negative isolation kit (Dynabeads 450; Dynal) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was more than 95% as assessed by fluorescence-activated cell sorting (FACS) analysis. Naïve $CD4^+$ T cells were produced by further treatment of $CD4^+$ T cells with an mAb to CD45RO antigen, followed by incubation with beads coated with goat antimouse IgG. Memory-type T cells were similarly produced by the treatment of cells with an mAb to CD45RA antigen. The purified responder cells ($1 \times 10^5$ well$^{-1}$) were plated in 96-well round-bottom tissue culture plates and macrophages or DCs were added to give the indicated APC/$CD4^+$ T-cell ratio. Supernatants of the cocultures were collected on day 4 and the concentration of cytokines was determined. In some cases, macrophages were treated with the indicated dose of exogenous rGM-CSF (Pepro Tech) in advance of infection with rBCGs. Further, macrophages were infected with rBCGs in the presence of neutralizing mAb to IL-10 (JES3-9D7; Rat IgG, BD PharMingen) or control normal rat IgG. Macrophages infected with BCGs were further costimulated with either rCD40L (Pepro Tech) or rIFN-γ (R&D Systems), and in some cases, the macrophages were stimulated with rIFN-γ in the presence of anti-IFN-γ receptor α chain (CD119) (GIR-208, mouse IgG1, BD PharMingen) or control normal mouse IgG. In other cases, macrophages infected with BCG-ΔUT in the presence of exogenous rIFN-γ were treated with either mAb to HLA-DR (L243, mouse IgG2a), CD86 (IT2.2, mouse IgG2b, BD PharMingen) or control normal mouse IgG, and subsequently cocultured with responder $CD4^+$ T cells. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit [OptEIA Human enzyme linked immunosorbent assay (ELISA) Set; BD Biosciences].

### Production of IL-12p70 and IL-1β by DCs

The ability of DCs to produce IL-12p70 and IL-1β on stimulation with BCG-Tokyo or BCG-ΔUT was assessed. The DCs were stimulated with BCGs at the indicated MOI for 24 h, and the concentration of these cytokines was quantified using the Opt EIA Human ELISA Set.

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### Animal studies

For inoculation into mice, BCG-Tokyo and BCG-ΔUT were cultured in Middlebrook 7H9 to log phase and stored at $10^8$ CFU mL$^{-1}$ at $-80\,^{\circ}$C. Before aliquots were used for inoculation, the concentration of viable bacilli was determined by plating cells on the Middlebrook 7H10 agar plate. Three 5-week-old C57BL/6J mice per group were inoculated intradermally with 0.1 mL phosphate-buffered saline (PBS) containing $1 \times 10^2$ or $1 \times 10^3$ BCG-Tokyo or BCG-ΔUT. The animals were kept under specific pathogen-free conditions and were supplied with sterilized food and water. Four weeks after injection, the spleens were removed, and the splenocytes were suspended at a concentration of $2 \times 10^6$ cells mL$^{-1}$ in culture medium, and stimulated with the indicated concentration of BCC or MLM in triplicate in 96-well round-bottomed microplates. The individual culture supernatants were collected 3 days after stimulation, and IFN-γ and IL-2 were measured using an OptEIA mouse ELISA set.

### Statistical analysis

The Student's *t*-test was applied to determine statistical differences.

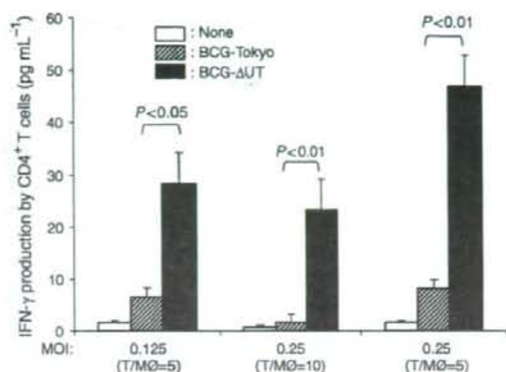


**Fig. 2.** Production of IFN-γ by CD4$^+$ T cells. Macrophages, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-Tokyo (parental BCG) or BCG-ΔUT at the indicated MOI, and cultured for another 2 days in the presence of rM-CSF. These macrophages were used as a stimulator of autologous CD4$^+$ T cells ($1 \times 10^5$ cells well$^{-1}$) at the indicated T-cell/macrophage ratio in a 4-day culture. A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

## Results

### Induction of the fusion of BCG-ΔUT-infected phagosomes with lysosomes

The efficacy with which BCG-ΔUT-infected phagosomes fused with lysosomes in macrophages was examined using confocal microscopy. Lysosomes were stained with anti-LAMP1 mAb after treatment of THP-1 cells with FITC-labeled BCG-Tokyo or BCG-ΔUT for 24 h. The parental BCG colocalized with lysosomes less efficiently than BCG-ΔUT (data not shown). Therefore, BCG-ΔUT may at least partially enhance the ability to induce phagosomal maturation.

### T-cell-stimulating activity of BCG-ΔUT

The activity of BCG-ΔUT to stimulate IFN-γ-producing CD4$^+$ T cells, when infected to macrophages, was assessed (Fig. 2). BCG-ΔUT-infected macrophages activated unseparated CD4$^+$ T cells to release IFN-γ substantially more efficiently than parent BCG-infected macrophages. Although BCG-ΔUT-infected macrophages also induced production of IL-2 from CD4$^+$ T cells (data not shown), the extent of IFN-γ ($< 50$ pg mL$^{-1}$) and IL-2 production was not as high as expected. Furthermore, BCG-ΔUT did not induce the activation of naïve CD4$^+$ T cells (data not shown). As the activation of T cells is largely influenced by the cytokine milieu, in which T cells and their stimulators are present, we determined the level of cytokines produced from macrophages on stimulation with BCG-ΔUT. BCG-ΔUT produced significantly more cytokines, such as IL-10, GM-CSF, TNFα and IL-1β, than the parental BCG (data not shown). It has been reported that IL-10 inhibits the APC-mediated activation of T cells (Granelli-Piperno *et al.*, 2004) and GM-CSF regulates the function of macrophages (Makino *et al.*, 2007). To examine the role of IL-10 on T-cell activation, macrophages were infected with BCGs in the presence of a neutralizing mAb to IL-10 (Fig. 3a). The IFN-γ production by stimulated CD4$^+$ T cells was not influenced by the treatment of macrophages with control IgG; however, a significantly higher level of IFN-γ was produced on treatment with the neutralizing mAb to IL-10. The up-regulation by IL-10 mAb treatment was observed in both BCG-Tokyo and BCG-ΔUT in a similar fashion. Furthermore, the pretreatment of macrophages with exogenous GM-CSF also significantly upregulated the antigen-presenting function of macrophages, although the effect of GM-CSF was more pronounced in BCG-ΔUT-infected macrophages (Fig. 3b).

Next, we phenotypically assessed the effect of BCG-ΔUT on macrophages (Fig. 4a). BCG-ΔUT induced enhanced expression of both CD14 and CD40 on macrophages compared with BCG-Tokyo. Based on these results, we treated BCG-infected macrophages with CD40L to examine its role as a costimulator of macrophages (Fig. 4b). The CD40L treatment upregulated the T-cell activation by BCG-



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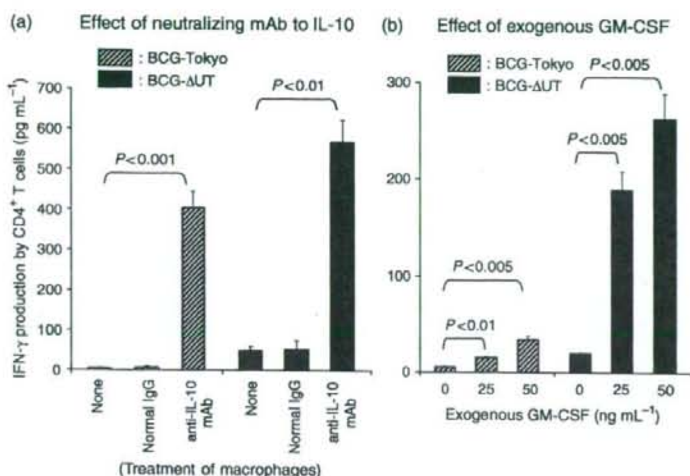


**Fig. 3.** Effect of IL-10 and GM-CSF on IFN-γ production. (a) Macrophages differentiated from monocytes by using rM-CSF were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 on day 5 of culture and cultured for another 2 days in the presence of rM-CSF. The BCG-infected macrophages were treated with neutralizing mAb to IL-10 or isotype-matched control IgG (10 μg mL$^{-1}$), and used as a stimulator of CD4$^+$ T cells, at a T-cell/macrophage ratio of 10 : 1, and cultured for another 4 days. The optimal concentration of mAb was determined in advance. (b) Macrophages obtained by 4 days of culture with rM-CSF were treated with the indicated dose of rGM-CSF. The macrophages pretreated with rGM-CSF were infected with BCG-Tokyo or BCG-ΔUT at an MOI of 0.25, cultured for another 2 days in the presence of rM-CSF used as a stimulator of CD4$^+$ T cells on day 8, at a T-cell/macrophage ratio of 10 : 1 (4 days of stimulation). A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's t-test.

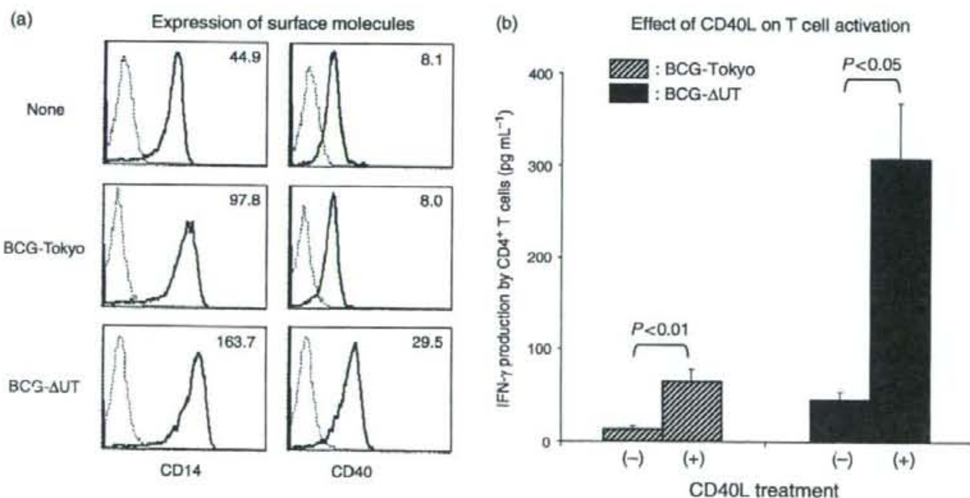


**Fig. 4.** (a) Expression of CD14 and CD40 molecules on macrophages. Macrophages produced by using rM-CSF were infected with BCGs at an MOI of 0.25, and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) IFN-γ production by CD4$^+$ T cells stimulated with BCG-infected macrophages. Macrophages differentiated from monocytes using rM-CSF were infected with BCGs at an MOI of 0.25 on day 5 of culture, further treated with CD40L (1 μg mL$^{-1}$) on day 6, and used as a stimulator of CD4$^+$ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's t-test.

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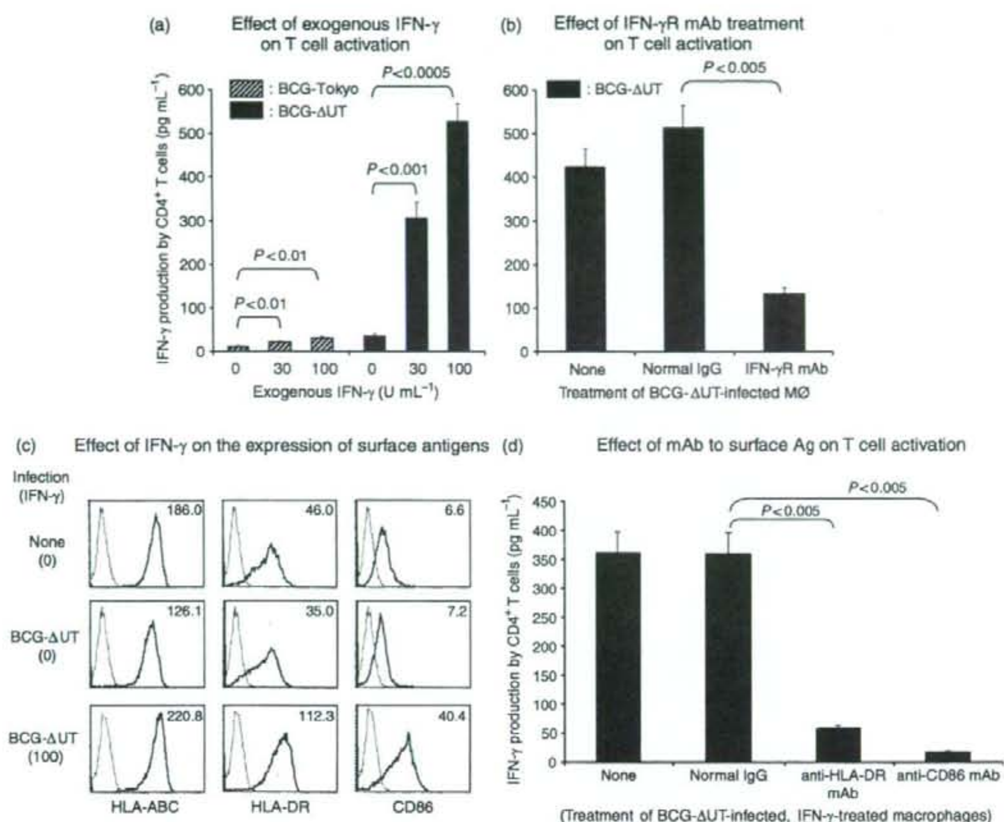


**Fig. 5.** (a) Effect of exogenous IFN-γ on CD4+ T-cell activation. Macrophages produced by 5 days of culture with rM-CSF from monocytes were infected with BCGs at an MOI of 0.25 and simultaneously treated with the indicated dose of exogenous IFN-γ. The macrophages were used as a stimulator of CD4+ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (b) Involvement of IFN-γ receptor in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL$^{-1}$) in the presence of mAb to IFN-γ receptor α-chain (CD119) or isotype matched control IgG (10 μg mL$^{-1}$), and cultured for another 2 days in the presence of rM-CSF. The macrophages were used as a stimulator of CD4+ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) Surface expression of various molecules on BCG-ΔUT-infected, IFN-γ-treated macrophages. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL$^{-1}$) and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (d) Involvement of surface antigens of BCG-ΔUT-infected, IFN-γ-stimulated macrophages in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), treated with exogenous IFN-γ (100 U mL$^{-1}$) and cultured for another 2 days in the presence of rM-CSF. These macrophages were cocultured with autologous CD4+ T cells at a T-cell/macrophage ratio of 10 : 1 in a 4-day culture in the presence of the indicated mAb (10 μg mL$^{-1}$). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

infected macrophages, but it more efficiently affected BCG-ΔUT-infected macrophages. Similarly, there was a significant difference between parent BCG and BCG-ΔUT in sensitivity to IFN-γ (Fig. 5a). However, other cytokines such as TNFα and IL-1β did not enhance the T-cell-stimulating activity of rBCG-infected macrophages. The IFN-γ treatment was effective against both BCG-Tokyo- and BCG-ΔUT-infected macrophages; however, more than a 10-fold increase in the production of IFN-γ from T cells was achieved only when BCG-ΔUT-infected macrophages


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were stimulated with exogenous IFN-γ. The optimal stimulation of T cells induced the production of more than 500 pg $mL^{-1}$ IFN-γ. The exogenous IFN-γ seems to contribute directly to the enhancement of APC function, as the IFN-γ-mediated enhancement was cancelled out by the pretreatment of BCG-ΔUT-infected macrophages with mAb to IFN-γ receptor α-chain (Fig. 5b). Furthermore, IFN-γ significantly enhanced the expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages (Fig. 5c), while the phenotypic alteration of BCG-Tokyo-infected macrophages by IFN-γ was minimum (data not shown). When BCG-ΔUT-infected, IFN-γ-treated macrophages were treated with mAb to either HLA-DR or CD86 in advance of being cocultured with $CD4^+$ T cells, IFN-γ production by the T cells was significantly inhibited, while normal murine IgG treatment did not have any effect (Fig. 5d).

### $CD4^+$ T-cell activation by BCG-ΔUT-infected DCs

As BCG-ΔUT significantly but less efficiently activated $CD4^+$ T cells through macrophages in the absence of costimulation, the potency of BCG-ΔUT-infected DCs as a T-cell activator was evaluated. Expression of surface molecules on DCs infected with either BCG-Tokyo or BCG-ΔUT was examined (Fig. 6a). Expression of HLA-ABC, HLA-DR, CD86 and CD83 was more significantly upregulated by the infection with BCG-ΔUT than with BCG-Tokyo. Higher levels of IL-12p70 and IL-1β were produced by BCG-ΔUT stimulation (Fig. 6b). Furthermore, we assessed whether BCG-ΔUT activated naïve and memory $CD4^+$ T cells through DCs by using various MOI titers and multiple T/DC ratios (Fig. 6c). IFN-γ levels were significantly higher following stimulation with BCG-ΔUT than with parent BCG in both naïve and memory $CD4^+$ T cells. Also, a higher level of CD40L was expressed on $CD4^+$ T cells after stimulation with BCG-ΔUT-infected DCs (data not shown). These results indicate that the infection of DCs with BCG-ΔUT alone was sufficient, as compared with macrophages which required costimulators to drive a strong T-cell response.

### Memory T-cell production by BCG-ΔUT

Another important aspect of using BCG as a vaccine is the production of memory T cells *in vivo*. We examined the response of splenic T cells obtained from BCG-infected C57BL/6 mice to mycobacterial recall antigen (Fig. 7). We used BCC as a recall antigen. At 4 weeks following infection, splenic T cells from BCG-ΔUT-infected mice produced more IFN-γ than those from mice infected with BCG-Tokyo by responding to BCC. The lymphocyte population producing IFN-γ was found to be $CD4^+$ T cells by intracellular staining (data not shown). Furthermore, upon stimulation with MLM, which contains immunodominant antigens of *M. leprae*, $CD4^+$ T cells from BCG-ΔUT-infected mice produced significantly higher levels of IFN-γ than those from uninfected or BCG-Tokyo-infected mice (Fig. 7).

## Discussion

To date, BCG is the only suitable vaccine against leprosy; however, its efficacy is quite limited. Overall efficacy in one meta-analysis was reported to be only 26% (Setia *et al.*, 2006). Several reasons might explain why BCG cannot block multiplication of *M. leprae* or inhibit the development of leprosy. The most important defect of BCG is that it is retained in phagosomes of macrophages, avoiding phagosomal acidification and hence interfering in the efficient fusion of BCG-containing phagosomes with lysosomes (Clements *et al.*, 1995; Reyrat *et al.*, 1995; Grode *et al.*, 2005). The lack of phagosome–lysosome fusion inhibits the trafficking of BCG-derived antigens through the major histocompatibility class (MHC) II pathway, which is enrolled for preferential stimulation of $CD4^+$ T cells, the most important cells involved in inhibition of *M. leprae* growth (Sendide *et al.*, 2004). Further, macrophages produce abundant amounts of IL-10 on infection with BCG, which, in turn, inhibits the activation of $CD4^+$ T cells (Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004).

In the present study, we constructed a recombinant BCG (BCG-ΔUT) that lacks a *urease* gene through allelic exchange of chromosomal DNA. As urease is involved in the maintenance of intraphagosomal pH at neutral (Grode *et al.*, 2005) or slightly alkaline values (Sendide *et al.*, 2004), lack of this enzyme may contribute to the induction of phagosomal acidification (Sendide *et al.*, 2004), thereby promoting the fusion of BCG-containing phagosomes with lysosomes. The efficient colocalization of BCG-ΔUT with lysosome was observed, leading us to expect an efficient enhancement of T-cell activation by BCG-ΔUT-infected macrophages. Previously, rBCG deficient in urease C was produced by a similar system and found to be superior to parental BCG in producing acidic conditions (pH 4.5–5.5) in BCG-infected phagosomes in murine macrophages (Reyrat *et al.*, 1995; Grode *et al.*, 2005). However, it was not demonstrated whether the rBCG deficient in urease C promoted the MHC class II trafficking pathway and actually activated human $CD4^+$ T cells through APCs. The newly constructed BCG-ΔUT lacked urease activity and *in vitro* studies confirmed that it could not degrade urea to ammonia. When BCG-ΔUT was infected to macrophages, it activated human $CD4^+$ T cells more efficiently than the parental BCG. However, the amount of IFN-γ released from the T cells was not as high as expected ($< 50$ pg $mL^{-1}$). These results suggest that

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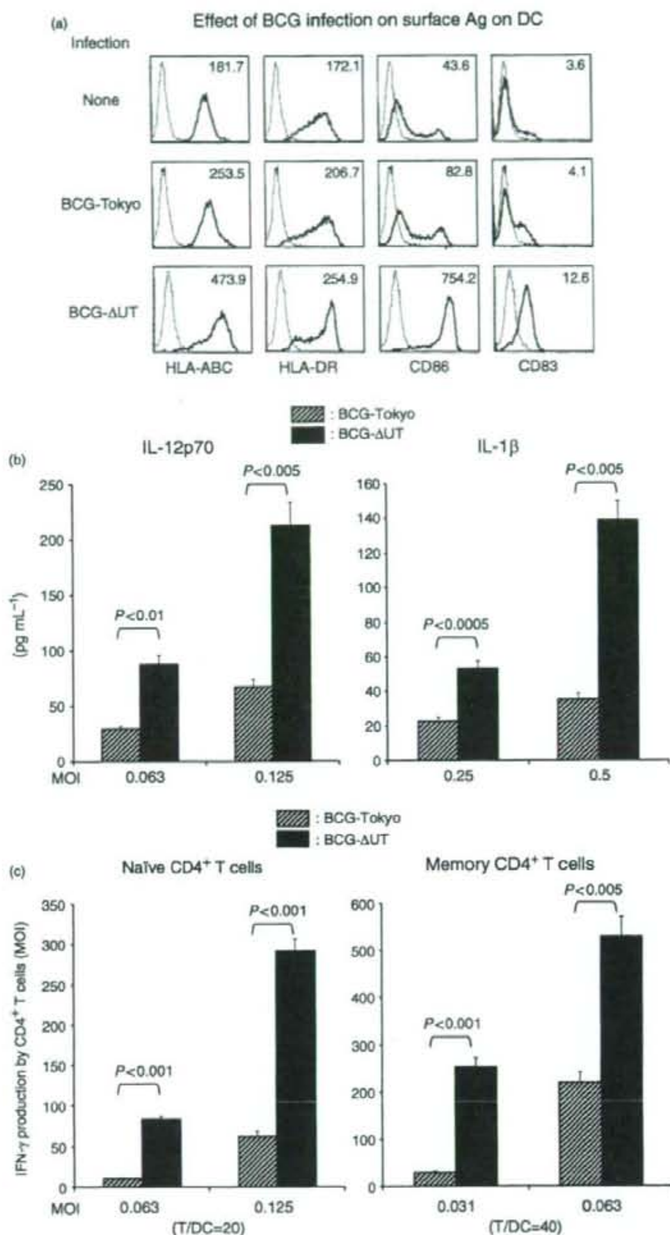


**Fig. 6.** (a) Expression of various molecules on BCG-infected DCs. Monocyte-derived immature DCs were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 5 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) Cytokine production from DCs stimulated by BCG. Monocyte-derived DCs from 4 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of either BCG-Tokyo or BCG-ΔUT for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) IFN-γ production by naïve CD4+ T cells and memory CD4+ T cells. DCs obtained from monocytes infected with either BCG-Tokyo or BCG-ΔUT were used as a stimulator of naïve and memory CD4+ T cells in a 4-day culture. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

improvement of intraphagosomal pH milieu for efficient phagosome–lysosome fusion was not sufficient for the induction of full T-cell activation as far as macrophages were concerned. Thus, we further searched for factors which might be helpful in inducing full activation of T cells. First, we examined the influence of endogenously produced IL-10, as abundant IL-10 was produced from macrophages by infection with BCG-ΔUT (data not shown). The neutralization of IL-10 from macrophages drastically enhanced T-cell activation (Fig. 3a). Furthermore, pretreatment of



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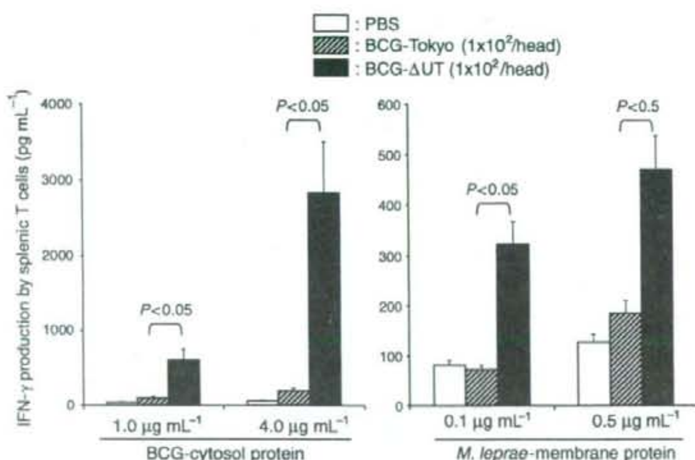


**Fig. 7.** IFN-γ production by splenic T cells obtained from C57BL/6 mice infected with BCG-Tokyo or BCG-ΔUT. Five-week-old C57BL/6 mice were infected with the indicated dose of BCG intradermally. Four weeks after the inoculation, splenocytes ($2 \times 10^5$ cells well$^{-1}$) were stimulated with the indicated dose of either BCG-derived cytosol protein or *Mycobacterium leprae*-derived membrane protein for 4 days. Assays were performed in triplicate for each mouse, and the results for three mice per group are given, expressed as the means ± SD. Representative results for two separate experiments are shown. Titers were statistically compared using Student's *t*-test.

macrophages with GM-CSF, which is normally produced from activated CD4$^+$ T cells, monocytes and macrophages (data not shown), and inhibits IL-10 production (Makino *et al.*, 2007), was also quite efficient in enhancing the BCG-ΔUT-mediated T-cell activity. Therefore, the unexpectedly weak activation of CD4$^+$ T cells by BCG-ΔUT seemed to be at least partly due to the immunosuppressive effect of IL-10. Secondly, we focused on the costimulating factors capable of actively up-regulating the T-cell-stimulating function of macrophages, and found that both CD40L and IFN-γ were quite efficient. It was previously reported that both CD40L and IFN-γ were needed to costimulate macrophages infected with *M. leprae* (Makino *et al.*, 2007); however, in the present study, the sole treatment of BCG-ΔUT-infected macrophages with either CD40L or IFN-γ was enough to confer a sufficient effect (Figs 4 and 5). The high sensitivity of BCG-ΔUT-infected macrophages to CD40L may be due to the ability of rBCG to induce greater expression of CD40 (Fig. 4a). The exogenous IFN-γ may contribute to increased production of IFN-γ from T cells by activating macrophages, as it enhanced the surface expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages, which facilitated antigen-specific T-cell activation. As reported, *M. leprae* is less sensitive to IFN-γ (Makino *et al.*, 2007), and also parental BCG was found to be clearly less sensitive to IFN-γ than BCG-ΔUT. These results indicate that each mycobacterium may have differential sensitivity to IFN-γ (Verreck *et al.*, 2004). Although the molecular mechanism responsible for the difference in sensitivity remains unexplained, it is well known that IFN-γ facilitates the digestion of intracellular mycobacteria in macrophages, and thus the following speculation may be possible: in the present system, the alteration of the pH milieu of BCG-containing phagosomes caused by the depletion of urease activity may help to establish circumstances where cell activation as well as enhanced trafficking of mycobacterial antigens to the surface by the MHC class II pathway can be induced by IFN-γ treatment. The urease gene of pathogenic mycobacteria may be a good target for combination immunotherapy/chemotherapy as urease depletion downregulates the growth of mycobacteria (data not shown) and upregulates the immunoactivity of intracellular digestion of bacteria in host cells.

In contrast to macrophages, DCs were highly activated by the sole infection with BCG-ΔUT in terms of phenotype and cytokine production, and BCG-ΔUT-infected DCs efficiently activated both naïve and memory CD4$^+$ T cells in the absence of additional costimulation. The activated T cells produced abundant amounts of both IFN-γ (Fig. 5c) and GM-CSF, and induced CD40L expression (data not shown). Therefore, DCs can inherently provide the critical factors needed by BCG-ΔUT-infected macrophages. As BCG infects both macrophages and DCs *in vivo*, we evaluated the efficacy of BCG-ΔUT as a T-cell activator by using C57BL/6 mice. BCG-ΔUT was superior to BCG-Tokyo in the production of murine memory CD4$^+$ T cells, which can respond to BCG-derived recall antigen and also proteins derived from pathogenic *M. leprae*. Just 100 BCG-ΔUT bacilli were sufficient to produce such memory T cells. These findings indicate that BCG-ΔUT convincingly stimulated CD4$^+$ T cells *in vivo*. As the C57BL/6 strain is a T helper (Th)1 response-prone mouse, further study using Th2 response-prone mice would provide further insight into how memory T cells are generated by inoculation with BCG-ΔUT.

Taking our data together, BCG-ΔUT is more potent than the parental BCG in the activation of macrophages, DCs and CD4$^+$ T cells. The depletion of urease from BCG may be useful in upregulating the potency of BCG as an immunostimulator.

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