CLINICAL AND VACCINE IMMUNOLOGY, Dec. 2008, p. 1755–1759
1556-6811/08/$08.00+0 doi:10.1128/CVI.00148-08
Copyright © 2008, American Society for Microbiology. All Rights Reserved.
Vol. 15, No. 12

Serological Diagnosis of Leprosy in Patients in Vietnam by Enzyme-Linked Immunosorbent Assay with *Mycobacterium leprae*-Derived Major Membrane Protein II[▽]

Masanori Kai,[1]* Nhu Ha Nguyen Phuc,[2] Thuy Huong Hoang Thi,[2] An Hoang Nguyen,[2] Yasuo Fukutomi,[1] Yumi Maeda,[1] Yuji Miyamoto,[1] Tetsu Mukai,[1] Tsuyoshi Fujiwara,[3] Tan Thanh Nguyen,[2] and Masahiko Makino[1]

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan[1]; Quyhoa National Leprosy & Dermato-Venereology Hospital, Ghenhrang District, Quynhon City, Binhdinh, Vietnam[2]; and Institute for Natural Science, Nara University, Nara 631-8502, Japan[3]

Received 21 April 2008/Returned for modification 13 June 2008/Accepted 12 October 2008

A serological diagnostic test using phenolic glycolipid-I (PGL-I) developed in the 1980s is commercially available, but the method is still inefficient in detecting all forms of leprosy. Therefore, more-specific and -reliable serological methods have been sought. We have characterized major membrane protein II (MMP-II) as a candidate protein for a new serological antigen. In this study, we evaluated the effectiveness of the enzyme-linked immunosorbent assay (ELISA) using the MMP-II antigen (MMP-II ELISA) for detecting antibodies in leprosy patients and patients' contacts in the mid-region of Vietnam and compared to the results to those for the PGL-I method (PGL-I ELISA). The results showed that 85% of multibacillary patients and 48% of paucibacillary patients were positive by MMP-II ELISA. Comparison between the serological tests showed that positivity rates for leprosy patients were higher with MMP-II ELISA than with PGL-I ELISA. Household contacts (HHCs) showed low positivity rates, but medical staff members showed comparatively high positivity rates, with MMP-II ELISA. Furthermore, monitoring of results for leprosy patients and HHCs showed that MMP-II is a better index marker than PGL-I. Overall, the epidemiological study conducted in Vietnam suggests that serological testing with MMP-II would be beneficial in detecting leprosy.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection, which sometimes leads to progressive peripheral nerve injury and systematic deformity (16, 30). Early detection of *M. leprae* infection and early start of treatment are key in avoiding deformities. Also, in order to decrease the incidence of new cases, it is important to find and treat the sources of the infection as soon as possible. Thus, early detection of these infected individuals who cannot be clinically diagnosed is critical (34). The diagnosis of leprosy is based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected patients. Recently, diagnostic methods for leprosy based on *M. leprae* DNA sequences have been developed (10, 20, 25). However, it is difficult to use these methods in developing countries which still have leprosy hot spot areas, because such methods require expensive machines and materials as well as skilled technicians. Although many developing countries have recently established laboratories for DNA-based diagnosis, it is harder to perform DNA tests than serodiagnostic tests. Thus, in countries where leprosy is endemic, diagnosis still relies on clinical observations and easy, inexpensive tests.

Serodiagnosis is generally accepted as the easiest way of diagnosing a disease. For leprosy serodiagnosis, the only antigen currently used is phenolic glycolipid I (PGL-I), which is supposedly specific to *M. leprae* (21, 26, 27). Since the identification of PGL-I in 1981 by Hunter and Brennan (14), a number of serological tools have been developed. Simple assays, such as the Serodia-Leprae method, a dipstick assay, and lateral flow tests based on the PGL-I antigen, have been used to detect leprosy patients in areas where leprosy is endemic (3, 15, 17, 32). However, these tests seem to be insufficient for detection of both multibacillary (MB) and paucibacillary (PB) patients, as well as for early diagnosis, and have not been used as widely as would be expected in field situations (6, 29). Therefore, we have begun the search for a more sensitive antigen. Major membrane protein II (MMP-II; encoded by the ML2038c gene, named *bfrA*, also known as bacterioferritin) was previously identified from the cell membrane fraction of *M. leprae* as an antigenic molecule capable of activating both antigen-presenting cells and T cells (19, 24). A homology search of the mycobacteria nucleotide database revealed that MMP-II is conserved between *M. leprae*, *M. tuberculosis*, and *M. avium*. The amino acid identity is about 86% among the three species. However, we have previously examined the role of MMP-II in the humoral responses of Japanese patients and showed that MMP-II could contribute to the specific serodetection of leprosy patients (18).

In the present study, we performed a serological test using serum samples collected in regions of leprosy endemicity in Vietnam and evaluated the use of MMP-II as an antigen for serodiagnosis of leprosy. We believe that identifying the appropriate antigens for serodiagnosis could facilitate the devel-

* Corresponding author. Mailing address: Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-391-8807. E-mail: mkai@nih.go.jp.
▽ Published ahead of print on 22 October 2008.

opment of simple diagnostic tests, like dip-stick assays, for use in developing countries.

MATERIALS AND METHODS

Serum samples. A total of 974 serum samples from various individuals, including in- and out-patients of Quyhoa National Leprosy & Dermato-Venereology Hospital (NDH), were obtained under informed consent. The sera were donated by 205 leprosy patients (163 patients undergoing treatment and 42 new patients), 428 household contacts (HHCs), 130 medical staff members, and 211 noncontact healthy individuals. Sera of leprosy patients and their contacts were taken at regional medical centers in the midregion of Vietnam, including those in the Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Gialai, Kontum, Daklak, and Daknong provinces, where the average prevalence rate is 0.17 (number of cases/10,000 persons) and the average detection rate is 2.13 (number of cases/10,000 persons). Among these provinces, Binhdinh, Ninhthuan, Gialai, and Kontum had hot spot areas. The medical staff members consisted of workers in Quyhoa NDH, including medical doctors, nurses, pharmacists, technicians, and helpers. Only the sera from medical staff members who were not HHCs of leprosy patients were used in this study. Sera were also obtained from healthy persons living in the Binhdinh province (n = 126) and the Longan province (n = 85), which are distantly located from each other. Out of 205 leprosy patients, 121 had MB leprosy and 84 had PB leprosy. We made the initial diagnosis according to the Ridley-Jopling classification system and classified patients as MB and PB types based on the WHO recommendation. In Vietnam, the *M. bovis* bacille Calmette-Guérin (BCG) vaccination against tuberculosis has been undertaken in earnest since 1976. Almost all medical staff personnel who donated their blood for this study were vaccinated with BCG.

MMP-II and PGL-I antigens. The MMP-II gene (ML2038c, or *bfrA*) was expressed in *Escherichia coli* as a fusion construct by using a pMAL-c2X expression vector (New England BioLabs) (18). Synthetic bovine serum albumin-conjugated trisaccharide-phenyl propionate for the detection of PGL-I antibodies was produced by our laboratory. The procedure for synthesis of the antigen is described elsewhere (12).

ELISAs for detection of antibodies. MaxiSorp (Nalge Nunc) microtiter plates were coated with 50 μl antigen solution (MMP-II [0.4 μg/ml] and PGL-I [0.2 μg/ml]) in carbonate-bicarbonate buffer (pH 9.4) and kept at 4°C overnight. The optimal concentrations of these antigens were determined in advance. The enzyme-linked immunosorbent assay (ELISA) protocol was performed as described previously (18). We measured anti-MMP-II immunoglobulin G (IgG) antibodies and anti-PGL-I IgM antibodies. Plate-to-plate variations in optical density (OD) readings were controlled for by using a common standard serum.

Monitoring. One hundred forty-eight leprosy patients have been monitored using MMP-II ELISA and PGL-I ELISA during their multidrug therapy (MDT) treatment since 2001. Twelve-month MDT for MB was carried out, and sampling was performed three to five times. Also, HHCs were monitored once every 3 or 6 months by both the MMP-II and the PGL-I ELISA methods from 2001 to 2004.

Statistics. The data were analyzed using a statistical software package (version 9.3.2.0; MedCalc software). A receiver operator characteristic (ROC) curve was drawn to calculate the cutoff levels (2). Additionally, the statistically significant differences between assays were confirmed by the chi-square test (28).

RESULTS

Comparison of the distribution of ELISA values between MMP-II and PGL-I. We focused on the distribution of ELISA values derived from MB leprosy patients and compared them to those from healthy individuals (Fig. 1). The cutoff OD_{405} value for anti-MMP-II antibody was defined as 0.103 (95% confidence interval, 85.2 to 93.7), and that for anti-PGL-I antibody was defined as 0.452 (95% CI, 85.2 to 93.7), by ROC curve analysis (MedCalc software) using OD titers from 211 healthy individuals and 205 leprosy patients. The distribution pattern of MMP-II ELISA values was quite different from that of PGL-I ELISA for healthy individuals. While the OD values of most healthy individuals were in the low range for MMP-II ELISA (Fig. 1A), the titers obtained by PGL-I ELISA showed a bell-shaped curve which was similar to that of MB leprosy patients (Fig. 1B). The PGL-I ELISA values for PB leprosy patients also showed a similar bell-shaped curve (data not shown).

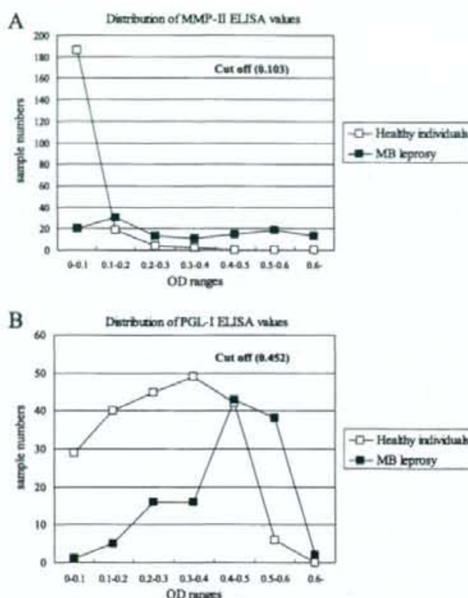

FIG. 1. Comparison of distributions of OD values in MB leprosy patients and normal individuals. (A) Distribution pattern of MMP-II ELISA values in patients and healthy individuals. (B) Distribution pattern of PGL-I ELISA values in patients and healthy individuals. The solid squares show the number of MB leprosy patients in each OD value range, and the open squares show the number of healthy individuals.

Detection rate of antibodies in sera of leprosy patients. Among the MB patients, 85.1% were positive by MMP-II ELISA and 57.0% were positive by PGL-I ELISA; 47.6% of PB patients were positive by MMP-II ELISA, and 20.2% were positive by PGL-I ELISA (Fig. 2). The MMP-II ELISA values for both MB and PB patients were significantly higher than the PGL-I ELISA values ($P < 0.001$) (Fig. 2). Patients undergoing treatment and new cases showed a similar difference (data not shown).

Seropositivity rates of contacts, medical staff members, and healthy volunteers. There was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals and HHCs (Fig. 3). Also, there was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals from different provinces, namely, Binhdinh and Longan (data not shown). In contrast, the medical staff showed a significantly higher rate of positivity by MMP-II ELISA (26.2%) than by PGL-I ELISA. The anti-MMP-II antibody positivity rate for the medical staff

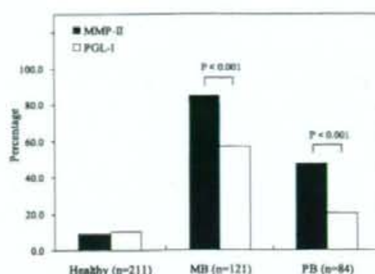

FIG. 2. Comparison of positivity rates of patients as determined by MMP-II and PGL-I ELISA. Black bars show percentages of healthy individuals and patients positive by MMP-II ELISA, and white bars show those for PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as P values.

was significantly higher than those for healthy individuals and HHCs.

Monitoring of HHCs. Previous studies suggested the usefulness of PGL-I ELISA in monitoring the effects of leprosy treatment (5, 8, 9, 22). Therefore, we monitored anti-MMP-II antibody titers in patients after treatment and compared them to anti-PGL-I antibody titers. Ninety-two MB and 56 PB patients were monitored. The anti-MMP-II antibody value of approximately 30% of monitored MB patients declined within 1 to 2 years after the start of treatment, in accordance with changes in bacterial index values (data not shown), although approximately 50% of MB patients showed no reduction in ELISA values and 20% of patients showed mild increases in value. Three representative samples of MB patients are shown in Fig. 4. Among PB patients, 18% of the monitored patients had reduced anti-MMP-II antibody titers. On the other hand, anti-PGL-I antibody titers were reduced approximately only 20% in both MB and PB patients during the monitoring period. Therefore, anti-MMP II antibody may reflect the efficacy of treatment similarly to or slightly better than anti-PGL-I antibody in some cases. Furthermore, 9 individuals out of 428

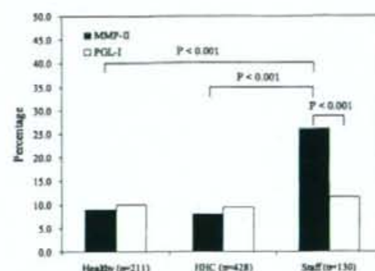

FIG. 3. Positivity rates of HHCs and medical staff members as determined by MMP-II and PGL-I ELISA. Black bars show percentages of HHCs and medical staff members positive by MMP-II ELISA, and white bars show those by PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as P values.

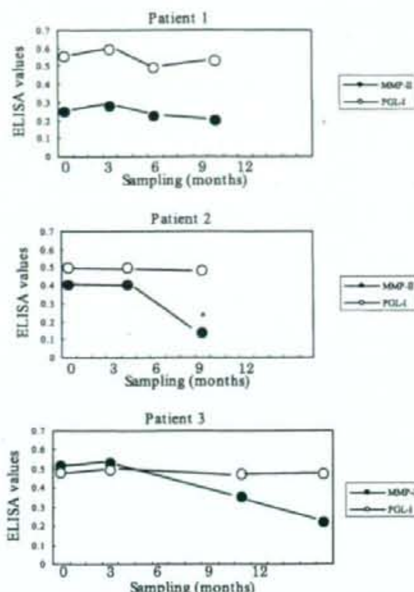

FIG. 4. Monitoring of three MB leprosy patients by MMP-II and PGL-I ELISAs. Three cases of monitored leprosy patients are shown. The closed circles show MMP-II ELISA values, and the open circles show PGL-I ELISA values. Note that the cutoff value for MMP-II is 0.103 and that of PGL-I is 0.452.

HHCs developed leprosy after several years of monitoring. Among the nine cases, two individuals had increasing antibody titers by MMP-II and/or PGL-I ELISA 1 year before manifesting clinical symptoms (data not shown). Patient HHC192 showed a prominent rise in anti-MMP-II antibody values during the asymptomatic period. Both patients developed MB leprosy. The other seven, whose antibody levels did not show an apparent increase during the observation period, developed PB leprosy.

DISCUSSION

Serodiagnosis is the easiest, cheapest, and least invasive diagnostic tool for infectious diseases. Currently, PGL-I is used as a specific antigen for *M. leprae*, but in practice, its sensitivity and specificity are not as high as expected, even though previous studies using stock sera reported that the detection rate for MB patients was more than 80% (1, 3, 4, 7). The present study involving Vietnamese leprosy patients indicated that there is a significant difference between MMP-II ELISA and PGL-I ELISA in detecting both MB and PB leprosy. The positivity rate of anti-MMP-II antibody for MB leprosy was approximately 85%, and that for PB leprosy was 48%; these titers were significantly higher than the titers obtained by PGL-I ELISA (57% and 20%, respectively). The detection rates obtained by

MMP-II ELISA were similar to those for a previous study using stock sera from Japanese leprosy patients (18). However, the positivity rates of anti-PGL-I antibody in the present study were significantly lower than those for the Japanese patients, although the same antigens for both MMP-II and PGL-I were used in the two studies.

There are several possible reasons why the sensitivity of PGL-I ELISA was low in the present study. One reason may be that some healthy Vietnamese individuals have high anti-PGL-I antibody titers. Although we could not conduct further detailed analysis on the subjects, these individuals might be highly exposed to *M. leprae*, and so their B lymphocytes might be repeatedly stimulated with *M. leprae*-derived antigens, including PGL-I. It seems quite difficult to discriminate the healthy individuals from MB or PB leprosy patients by PGL-I ELISA, as shown in Fig. 1. Furthermore, we concluded that a reasonable cutoff point for PGL-I ELISA was an OD_{405} of 0.452, as deduced from Fig. 1 and the ROC values, but this resulted in lower sensitivity. The difference in sensitivity between PGL-I ELISA and MMP-II ELISA may also be due to differences in the biochemical features of the antigens. PGL-I is a glycolipid component, and as such, it might be retained in some infected cells for a long time after the initial exposure (13, 33). This speculation is supported by previous reports showing that healthy individuals residing in areas where leprosy is endemic had high anti-PGL-I antibody titers, and *M. leprae* DNA was recovered by PCR from the nasal swabs of these individuals (31, 32). Also, it has been reported that the usefulness of PGL-I-based tests for early diagnosis is limited, since 7 to 10% of individuals testing positive do not develop the disease (14).

In contrast, MMP-II is a protein antigen and is considered to be one of the immunodominant antigens of *M. leprae* (19). Therefore, in individuals who have been exposed to *M. leprae* but have not developed leprosy, antigen-presenting cells expressing MMP-II might feasibly be eliminated from the body by immune cells such as cytotoxic T lymphocytes and thus lack the ability to produce anti-MMP-II antibodies through antigen-presenting-cell-dependent mechanisms. These speculations seem to be supported by our present observations with sera from patients monitored over time. Anti-MMP-II antibody titers of MB patients declined earlier than PGL-I titers with MDT treatment, indicating the disappearance of MMP-II antigens, while no apparent reduction in PGL-I antigens was observed during the 12 months of observation (Fig. 4). Furthermore, in one case the anti-MMP-II antibody titer increased drastically before manifestation of clinically apparent leprosy (data not shown).

Medical staff members (n = 130) showed a high positivity rate by MMP-II ELISA, compared with healthy individuals or HHCs. These medical staff members were mostly BCG vaccinated, as were the HHCs. Therefore, it seems that BCG vaccination has no effect on anti-MMP-II antibody titers. Although we could not determine a conclusive reason for the high positivity rate, these medical personnel may be repeatedly exposed to *M. leprae* in hospitals. However, we cannot eliminate the possibility that they have produced the antibody in response to exposure to other mycobacteria, since the MMP-II protein is conserved in other pathogenic mycobacterial species, such as *M. tuberculosis* and *M. avium*, though the staff members with high anti-MMP-II antibody titers did not manifest any clinical signs or features indicating infection with other mycobacteria. We tried to perform nested PCR using the *M. leprae*-specific repetitive element for DNA extracted from nasal swabs of some hospital staff members (n = 25). However, because the sampling dates for the serological test and the PCR test were not coordinated, we could not come to a definite conclusion. Nevertheless, we were surprised to find that ≅40% (n = 25) of the nasal swab samples were positive (data not shown). As for tuberculosis, it is said that one-third of the world population is infected with *M. tuberculosis*. The same may be the case with leprosy, although further studies are needed with larger populations, including medical staff members as well as contacts and noncontacts of leprosy.

Taken together, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool in combination with other clinical diagnostic methods and may also be useful in monitoring disease activity. Furthermore, in this study the correlation between MMP-II and PGL-I was low, with a correlation coefficient among the 205 leprosy patients of only 0.63. If both PGL-I and MMP-II antibodies could be measured simultaneously, the sensitivity of the assay system could be increased. Considering that PGL-I is a sugar antigen (eliciting IgM antibodies) and MMP-II is a protein antigen (eliciting IgG antibodies), assaying for a combination of these antibodies could lead to more-accurate detection of leprosy in the field.

ACKNOWLEDGMENTS

This research was supported in part by Health Sciences research grants for research on emerging and reemerging infectious diseases; by an international cooperation research grant (topic code 18C4) from the Ministry of Health, Labor and Welfare of Japan; and by Quyhoa NDH.

REFERENCES

1. **Agis, F., P. Schlich, J. L. Cartel, C. Guidi, and M. A. Bach.** 1988. Use of anti-*M. leprae* phenolic glycolipid-I antibody detection for early diagnosis and prognosis of leprosy. Int. J. Lepr. Other Mycobact. Dis. **56**:527–535.
2. **Beck, J. R., and E. K. Schultz.** 1986. The use of relative operating characteristic (ROC) curves in test performance evaluation. Arch. Pathol. Lab. Med. **110**:13–20.
3. **Bührer-Sékula, S., H. L. Smits, G. C. Gussenhoven, J. van Leeuwen, S. Amador, T. Fujiwara, P. R. Klatser, and L. Oskam.** 2003. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. J. Clin. Microbiol. **41**:1991–1995.
4. **Cartel, J. L., S. Chanteau, J. P. Boutin, R. Plichart, P. Richez, J. F. Roux, and J. H. Grosset.** 1990. Assessment of anti-phenolic glycolipid-I IgM levels using an ELISA for detection of *M. leprae* infection in populations of the South Pacific Islands. Int. J. Lepr. Other Mycobact. Dis. **58**:512–517.
5. **Chanteau, S., J. L. Cartel, P. Celerier, R. Plichart, S. Desforges, and J. Roux.** 1989. PGL-I antigen and antibody detection in leprosy patients: evolution under chemotherapy. Int. J. Lepr. Other Mycobact. Dis. **57**:735–743.
6. **Chanteau, S., P. Glaziou, C. Plichart, P. Luquiaud, R. Plichart, J. F. Faucher, and J. L. Cartel.** 1993. Low predictive value of PGL-I serology for the early diagnosis of leprosy in family contacts: results of a 10-year prospective field study in French Polynesia. Int. J. Lepr. Other Mycobact. Dis. **61**:533–541.
7. **Chaturvedi, V., S. Sinha, B. K. Girdhar, and U. Sengupta.** 1991. On the value of sequential serology with a *Mycobacterium leprae*-specific antibody competition ELISA in monitoring leprosy chemotherapy. Int. J. Lepr. Other Mycobact. Dis. **59**:32–40.
8. **Cho, S. N., R. V. Cellona, T. T. Fajardo, Jr., R. M. Abalos, E. C. dela Cruz, G. P. Walsh, J. D. Kim, and P. J. Brennan.** 1991. Detection of phenolic glycolipid-I antigen and antibody in sera from new and relapsed lepromatous patients treated with various drug regimens. Int. J. Lepr. Other Mycobact. Dis. **59**:25–31.
9. **Cho, S. N., R. V. Cellona, L. G. Villahermosa, T. T. Fajardo, Jr., M. V. Balagon, R. M. Abalos, E. V. Tan, G. P. Walsh, J. D. Kim, and P. J. Brennan.** 2001. Detection of phenolic glycolipid I of *Mycobacterium leprae* in sera from

leprosy patients before and after start of multidrug therapy. Clin. Diagn. Lab. Immunol. **8**:138–142.

10. **Donoghue, H. D., J. Holton, and M. Spigelman.** 2001. PCR primers that can detect low levels of *Mycobacterium leprae* DNA. J. Med. Microbiol. **50**:177–182.
11. Reference deleted.
12. **Fujiwara, T., S. W. Hunter, S. N. Cho, G. O. Aspinall, and P. J. Brennan.** 1984. Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. Infect. Immun. **43**:245–252.
13. **Gelber, R. H., F. Li, S. N. Cho, S. Byrd, K. Rajagopalan, and P. J. Brennan.** 1989. Serum antibodies to defined carbohydrate antigens during the course of treated leprosy. Int. J. Lepr. Other Mycobact. Dis. **57**:744–751.
14. **Hunter, S. W., and P. J. Brennan.** 1981. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147**:728–735.
15. **Izumi, S., T. Fujiwara, M. Ikeda, Y. Nishimura, K. Sugiyama, and K. Kawatsu.** 1990. Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. J. Clin. Microbiol. **28**:525–529.
16. **Job, C. K.** 1989. Nerve damage in leprosy. Int. J. Lepr. Other Mycobact. Dis. **57**:532–539.
17. **Kai, M., Y. Maeda, S. Maeda, Y. Fukutomi, K. Kobayashi, Y. Kashiwabara, M. Makino, M. A. Abbasi, M. Z. Khan, and P. A. Shah.** 2004. Active surveillance of leprosy contacts in country with low prevalence rate. Int. J. Lepr. Other Mycobact. Dis. **72**:50–53.
18. **Maeda, Y., T. Mukai, M. Kai, Y. Fukutomi, H. Nomaguchi, C. Abe, K. Kobayashi, S. Kitada, R. Maekura, I. Yano, N. Ishii, T. Mori, and M. Makino.** 2007. Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy. FEMS Microbiol. Lett. **272**:202–205.
19. **Maeda, Y., T. Mukai, J. Spencer, and M. Makino.** 2005. Identification of an immunomodulating agent from *Mycobacterium leprae*. Infect. Immun. **73**:2744–2750.
20. **Martinez, A. N., C. F. P. C. Britto, J. A. C. Nery, E. P. Sampaio, M. R. Jardim, E. N. Sarno, and M. O. Moraes.** 2006. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. J. Clin. Microbiol. **44**:3154–3159.
21. **Meeker, H. C., W. R. Levis, E. Sersen, G. Schuller-Levis, P. J. Brennan, and T. M. Buchanan.** 1986. ELISA detection of IgM antibodies against phenolic glycolipid-I in the management of leprosy: a comparison between laboratories. Int. J. Lepr. Other Mycobact. Dis. **54**:530–539.
22. **Meeker, H. C., G. Schuller-Levis, F. Fusco, M. A. Giardina-Becket, E. Sersen, and W. R. Levis.** 1990. Sequential monitoring of leprosy patients with serum antibody levels to phenolic glycolipid-I, a synthetic analog of phenolic glycolipid-I, and mycobacterial lipoarabinomannan. Int. J. Lepr. Other Mycobact. Dis. **58**:503–511.
23. Reference deleted.
24. **Pessolani, M. C., D. R. Smith, B. Rivoire, J. McCormick, S. A. Hefta, S. T. Cole, and P. J. Brennan.** 1994. Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. J. Exp. Med. **180**:319–327.
25. **Phetsuksiri, B., J. Rudeeaneksin, P. Supapkul, S. Wachapong, K. Mahotarn, and P. J. Brennan.** 2006. A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. FEMS Immunol. Med. Microbiol. **48**:319–328.
26. **Schuring, R. P., F. J. Moet, D. Pahan, J. H. Richardus, and L. Oskam.** 2006. Association between anti-PGL-I IgM and clinical and demographic parameters in leprosy. Lepr. Rev. **77**:343–355.
27. **Sekar, B., R. N. Sharma, G. Leelabai, D. Anandan, B. Vasanthi, G. Yusuff, M. Subramanian, and M. Jayasheela.** 1993. Serological response of leprosy patients to *Mycobacterium leprae* specific and mycobacteria specific antigens: possibility of using these assays in combinations. Lepr. Rev. **64**:15–24.
28. **Siegel, S., and N. J. Castellan, Jr.** 1988. Non-parametric statistics for the behavioral sciences, 2nd edition. McGraw-Hill, New York, NY.
29. **Soebono, H., and P. R. Klatser.** 1991. A seroepidemiological study of leprosy in high- and low-endemic Indonesian villages. Int. J. Lepr. Other Mycobact. Dis. **59**:416–425.
30. **Stoner, G. L.** 1979. Importance of the neural predilection of *Mycobacterium leprae* in leprosy. Lancet **ii**(8150):994–996.
31. **van Beers, S., M. Hatta, and P. R. Klatser.** 1999. Seroprevalence rates of antibodies to phenolic glycolipid-I among school children as an indicator of leprosy endemicity. Int. J. Lepr. Other Mycobact. Dis. **67**:243–249.
32. **van Beers, S., S. Izumi, B. Madjid, Y. Maeda, R. Day, and P. R. Klatser.** 1994. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int. J. Lepr. Other Mycobact. Dis. **62**:1–9.
33. **Verhagen, C., W. Faber, P. Klatser, A. Buffing, B. Naafs, and P. Das.** 1999. Immunohistological analysis of in situ expression of mycobacterial antigens in skin lesions of leprosy patients across the histopathological spectrum. Association of Mycobacterial lipoarabinomannan (LAM) and *Mycobacterium leprae* phenolic glycolipid-I (PGL-I) with leprosy reactions. Am. J. Pathol. **154**:1793–1804.
34. **World Health Organization.** 2007. Global leprosy situation. Wkly. Epidemiol. Rec. **82**:225–232.

RESEARCH ARTICLE

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

Tetsu Mukai, Yumi Maeda, Toshiki Tamura, Yuji Miyamoto & Masahiko Makino

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Correspondence: Masahiko Makino, Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Tel.: +81 42 391 8059; fax: +81 42 391 8212; e-mail: mmaki@nih.go.jp

Received 22 November 2007; revised 11 February 2008; accepted 15 February 2008. First published online 9 April 2008.

DOI:10.1111/j.1574-695X.2008.00407.x

Editor: Patrick Brennan

Keywords
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

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

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^2155) 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).

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

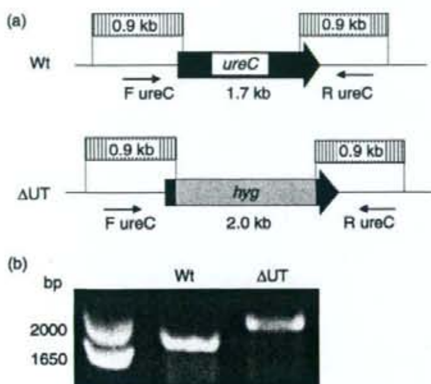

Fig. 1. Disruption of the *ureC* gene. (a) Schematic diagram of the *ureC* region on the chromosome of the wild-type *Mycobacterium bovis* BCG Tokyo strain and its gene disruptant, ΔUT. The shaded boxes indicate the regions included in the recombinant phage for gene disruption. The black arrow represents 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 1×10^4 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 (1×10^5 $well^{-1}$) 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.

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

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 °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 1×10^2 or 1×10^3 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 2×10^6 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.

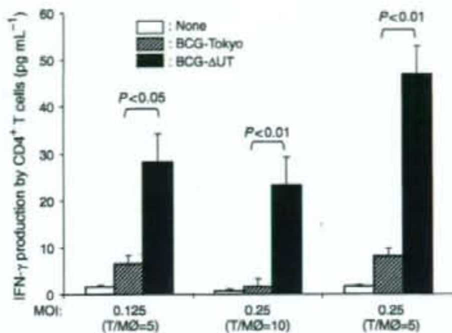

Fig. 2. Production of IFN-γ by CD4$^+$ T cells. Macrophages, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-Tokyo (parental BCG) or BCG-ΔUT at the indicated MOI, and cultured for another 2 days in the presence of rM-CSF. These macrophages were used as a stimulator of autologous CD4$^+$ T cells (1×10^5 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-

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

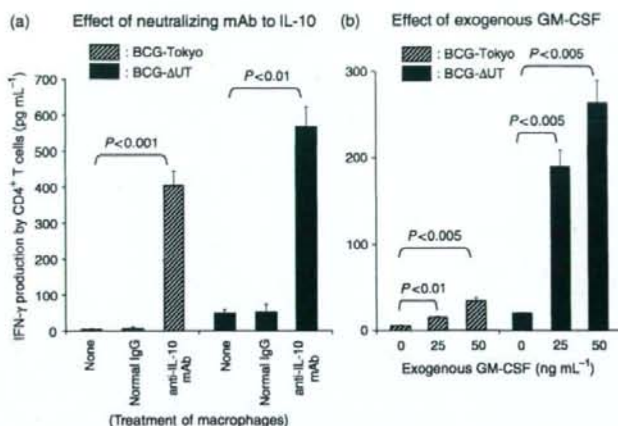

Fig. 3. Effect of IL-10 and GM-CSF on IFN-γ production. (a) Macrophages differentiated from monocytes by using rM-CSF were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 on day 5 of culture and cultured for another 2 days in the presence of rM-CSF. The BCG-infected macrophages were treated 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.

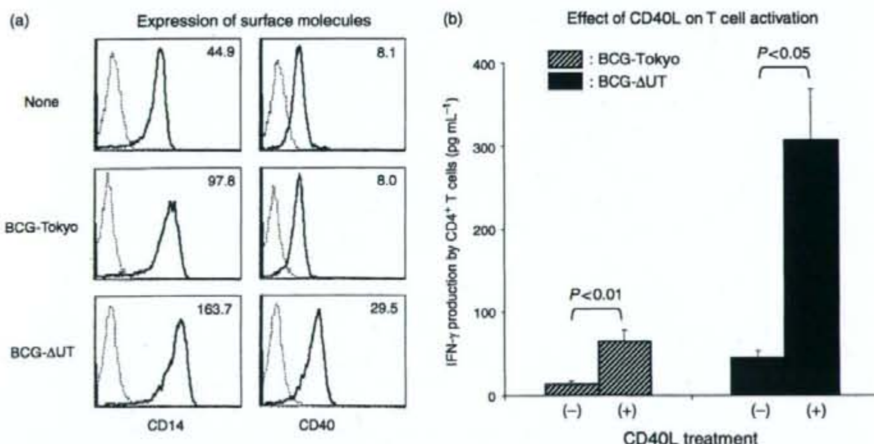

Fig. 4. (a) Expression of CD14 and CD40 molecules on macrophages. Macrophages produced by using rM-CSF were infected with BCGs at an MOI of 0.25, and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control 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.

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

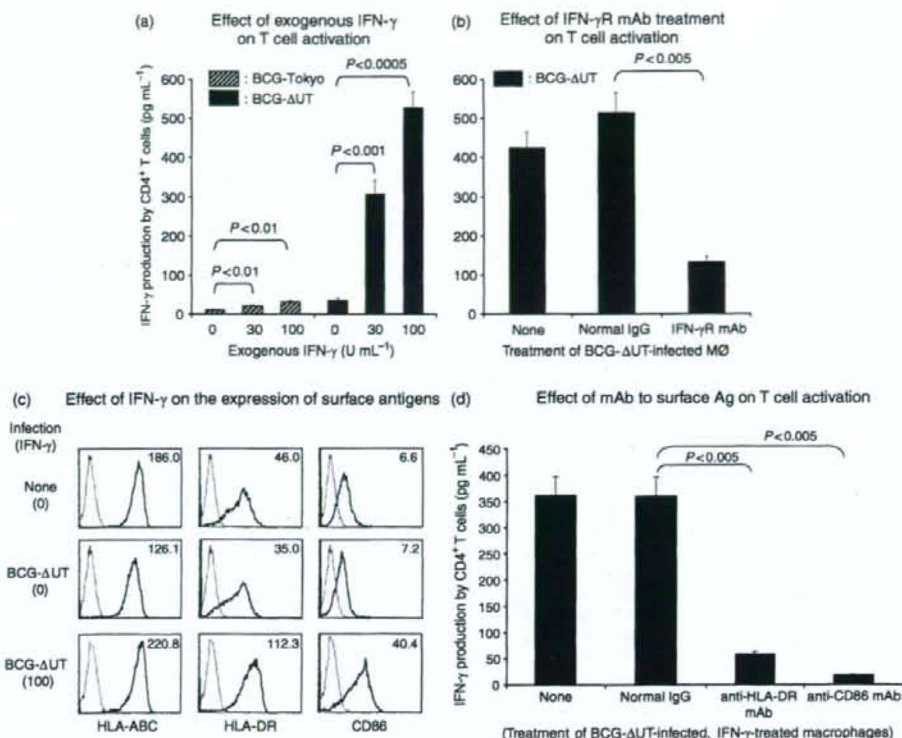

Fig. 5. (a) Effect of exogenous IFN-γ on CD4⁺ T-cell activation. Macrophages produced by 5 days of culture with rM-CSF from monocytes were infected with BCGs at an MOI of 0.25 and simultaneously treated with the indicated dose of exogenous IFN-γ. The macrophages were used as a stimulator of CD4⁺ T cells (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

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

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

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

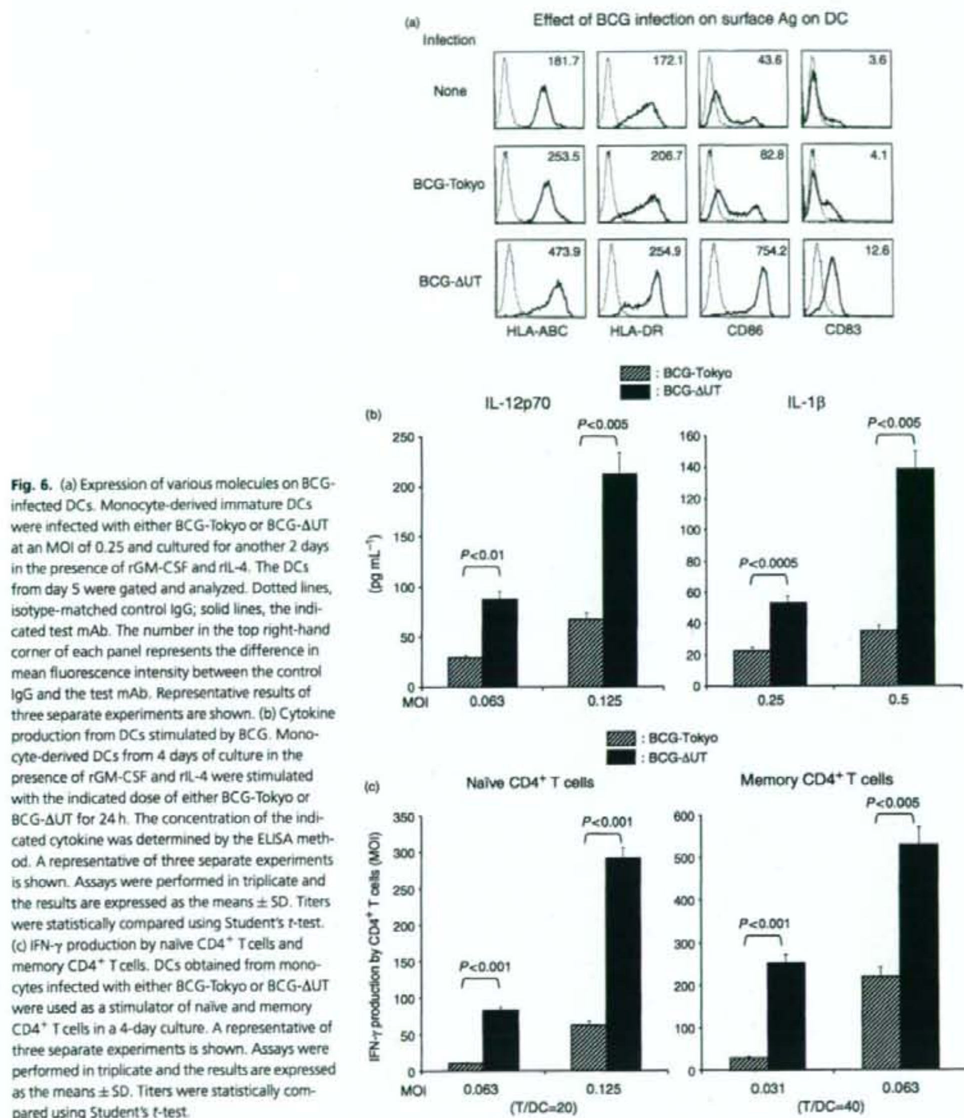

Fig. 6. (a) Expression of various molecules on BCG-infected DCs. Monocyte-derived immature DCs were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 5 were gated and analyzed. Dotted lines, isotype-matched 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

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

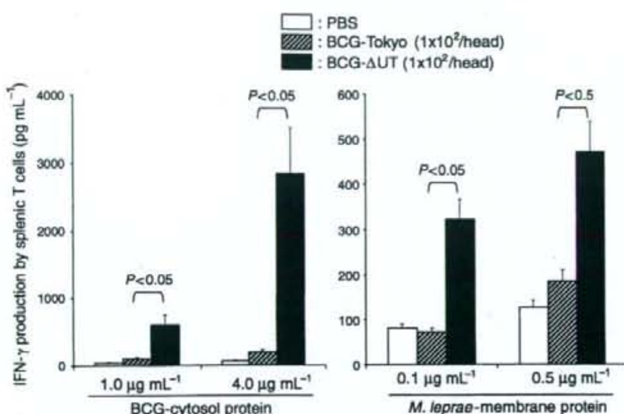

Fig. 7. IFN-γ production by splenic T cells obtained from C57BL/6 mice infected with BCG-Tokyo or BCG-ΔUT. Five-week-old C57BL/6 mice were infected with the indicated dose of BCG intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells well^{-1}) were stimulated with the indicated dose 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.

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Acknowledgements

We acknowledge the contribution of Ms N. Makino in the preparation of the manuscript. We thank Ms Y. Harada for her technical support and the Japanese Red Cross Society for kindly providing blood from healthy donors. We also thank Professor William R. Jacobs, Jr, Howard Hughes Medical Institute, for providing the plasmids, pYUB854 and phAE87. This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan.

References

Andersen P & Doherty TM (2005) The success and failure of BCG-implications for a novel tuberculosis vaccine. *Nat Rev Microbiol* **3**: 656–662.

Bardarov S, Bardarov S Jr, Pavelka MS Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G & Jacobs WR Jr (2002) Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis*. *Microbiology* **148**: 3007–3017.

Charles C & Shepard MD (1960) The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. *J Exp Med* **112**: 445–454.

Clements DL, Lee BY & Horwitz MA (1995) Purification, characterization, and genetic-analysis of *Mycobacterium tuberculosis* urease, a potentially critical determinant of host-pathogen interaction. *J Bacteriol* **177**: 5644–5652.

Dockrell HM, Young SK, Britton K *et al.* (1996) Induction of Th1 cytokine responses by mycobacterial antigens in leprosy. *Infect Immun* **64**: 4385–4389.

Dye C, Watt CJ, Bleed DM, Hosseini SM & Raviglione MC (2005) Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. *JAMA* **293**: 2767–2775.

Granelli-Piperno A, Golebiowska A, Trumpfheller C, Siegal FP & Steinman RM (2004) HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *Proc Natl Acad Sci USA* **101**: 7669–7674.

Grode L, Seiler P, Baumann S *et al.* (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. *J Clin Investig* **115**: 2472–2479.

Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M & Makino M (2002) *Mycobacterium leprae* infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. *Infect Immunity* **70**: 5167–5176.

Honerzu Bentrup K & Russell DG (2001) Mycobacterial persistence: adaptation to a changing environment [review]. *Trends Microbiol* **9**: 597–605.

Job CK (1989) Nerve damage in leprosy. *Int J Lepr Other Mycobact Dis* **57**: 532–539.

Kai M, Maeda Y, Maeda S, Fukutomi Y, Kobayashi K, Kashiwabara Y, Makino M, Abbasi MA, Khan MZ & Shah PA (2004) Active surveillance of leprosy contacts in country with low prevalence rate. *Int J Lepr Other Mycobact Dis* **72**: 50–53.

Kaufmann SHE (2005) Introduction. Rational vaccine development against tuberculosis: "those who don't remember the past are condemned to repeat it". *Microbes Infect* **7**: 897–898.

Kaufmann SHE (2006) Envisioning future strategies for vaccination against tuberculosis. *Nat Rev Immunol* **6**: 699–704.

Kaufmann SHE & McMichael AJ (2005) Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat Med* **11**: S33–S44.

Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, Hashimoto K, Kimura H, Kobayashi K & Kashiwabara Y (2001) Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother* **45**: 3635–3639.

Maeda Y, Gidoh M, Ishii N, Mukai C & Makino M (2003) Assessment of cell mediated immunogenicity of *Mycobacterium leprae*-derived antigens. *Cell Immunol* **222**: 69–77.

Makino M & Baba M (1997) A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. *Scand J Immunol* **45**: 618–622.

Makino M, Shimokubo S, Wakamatsu S, Izumo S & Baba M (1999) The role of human T-lymphotropic virus type 1 (HTLV-1)-infected dendritic cells in the development of HTLV-1-associated myelopathy/tropical spastic paraparesis. *J Virol* **73**: 4575–4581.

Makino M, Maeda Y, Fukutomi Y & Mukai T (2007) Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. *Microbes Infect* **9**: 70–77.

McDermott-Lancaster RD, Ito T, Kohsaka K, Guelpa-Lauras CC & Grosset JH (1987) Multiplication of *Mycobacterium leprae* in the nude mouse, and some applications of nude mice to experimental leprosy. *Int J Lepr Other Mycobact Dis* **55**: 889–895.

Miyamoto Y, Mukai T, Takeshita F, Nakata N, Maeda Y, Kai M & Makino M (2004) Aggregation of mycobacteria caused by disruption of fibronectin-attachment protein-encoding gene. *FEMS Microbiol Lett* **236**: 227–234.

Miyamoto Y, Mukai T, Nakata N, Maeda Y, Kai M, Naka T, Yano I & Makino M (2006) Identification and characterization of the genes involved in glycosylation pathways of mycobacterial glycopeptidolipid biosynthesis. *J Bacteriol* **188**: 86–95.

Mochida-Nishimura K, Akagawa KS & Rich EA (2001) Interleukin-10 contributes development of macrophage suppressor activities by macrophage colony-stimulating factor, but not by granulocyte-macrophage colony-stimulating factor. *Cell Immunol* **214**: 81–88.

Orme IM, Roberts AD, Griffin JP & Abrams JS (1993) Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. *J Immunol* **151**: 518–525.

Reyrat JM, Berthet FX & Gicquel B (1995) The urease locus of *Mycobacterium tuberculosis* and its utilization for the

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

demonstration of allelic exchange in *Mycobacterium bovis* bacillus Calmette-Guérin. *Proc Natl Acad Sci USA* **92**: 8768–8772.

Schaible UE, Sturgill-Koszycki S, Schlesinger PH & Russell DG (1998) Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. *J Immunol* **160**: 1290–1296.

Sendide K, Degmane AE, Reyrat JM, Talal A & Hmama Z (2004) *Mycobacterium bovis* BCG urease attenuates major histocompatibility complex class II trafficking to the macrophage cell surface. *Infect Immun* **72**: 4200–4209.

Setia MS, Steinmaus C, Ho CH & Rutherford GW (2006) The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect Dis* **6**: 162–170.

Stoner GL (1979) Importance of the neural predilection of *Mycobacterium leprae* in leprosy. *Lancet* **2**: 994–996.

Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R & Ottenhoff TH (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci USA* **101**: 4560–4565.

Wakamatsu S, Makino M, Tei C & Baba M (1999) Monocyte-driven activation-induced apoptotic cell death of human T-lymphotropic virus type I-infected T cells. *J Immunol* **163**: 3914–3919.

World Health Organization. (2006) *Fact Sheet No. 104*, Rev March, http://www.who.int/mediacentre/factsheets/fs104/en

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

JOURNAL OF BACTERIOLOGY, May 2008, p. 3613–3621
0021-9193/08/$08.00+0 doi:10.1128/JB.01850-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.
Vol. 190, No. 10

Structural Analysis and Biosynthesis Gene Cluster of an Antigenic Glycopeptidolipid from *Mycobacterium intracellulare*▽†

Nagatoshi Fujiwara,[1]* Noboru Nakata,[2] Takashi Naka,[1,3] Ikuya Yano,[3] Matsumi Doe,[4] Delphi Chatterjee,[5] Michael McNeil,[5] Patrick J. Brennan,[5] Kazuo Kobayashi,[6] Masahiko Makino,[2] Sohkichi Matsumoto,[1] Hisashi Ogura,[7] and Shinji Maeda[8]

Department of Host Defense[1] and Virology,[7] Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan; Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002, Japan[2]; Japan BCG Laboratory, Tokyo 204-0022, Japan[3]; Department of Chemistry, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan[4]; Department of Microbiology, Immunology and Pathology, Colorado State University, Colorado 80523[5]; Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[6]; and Molecular Epidemiology Division, Mycobacterium Reference Center, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo 204-8533, Japan[8]

Received 24 November 2007/Accepted 1 March 2008

***Mycobacterium avium*-*Mycobacterium intracellulare* complex (MAC) is the most common isolate of nontuberculous mycobacteria and causes pulmonary and extrapulmonary diseases. MAC species can be grouped into 31 serotypes by the epitopic oligosaccharide structure of the species-specific glycopeptidolipid (GPL) antigen. The GPL consists of a serotype-common fatty acyl peptide core with 3,4-di-*O*-methyl-rhamnose at the terminal alaninol and a 6-deoxy-talose at the *allo*-threonine and serotype-specific oligosaccharides extending from the 6-deoxy-talose. Although the complete structures of 15 serotype-specific GPLs have been defined, the serotype 16-specific GPL structure has not yet been elucidated. In this study, the chemical structure of the serotype 16 GPL derived from *M. intracellulare* was determined by using chromatography, mass spectrometry, and nuclear magnetic resonance analyses. The result indicates that the terminal carbohydrate epitope of the oligosaccharide is a novel *N*-acyl-dideoxy-hexose. By the combined linkage analysis, the oligosaccharide structure of serotype 16 GPL was determined to be 3-2′-methyl-3′-hydroxy-4′-methoxy-pentanoyl-amido-3,6-dideoxy-β-hexose-(1→3)-4-*O*-methyl-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→2)-6-deoxy-α-L-talose. Next, the 22.9-kb serotype 16-specific gene cluster involved in the glycosylation of oligosaccharide was isolated and sequenced. The cluster contained 17 open reading frames (ORFs). Based on the similarity of the deduced amino acid sequences, it was assumed that the ORF functions include encoding three glycosyltransferases, an acyltransferase, an aminotransferase, and a methyltransferase. An *M. avium* serotype 1 strain was transformed with cosmid clone no. 253 containing *gtfB*-*drrC* of *M. intracellulare* serotype 16, and the transformant produced serotype 16 GPL. Together, the ORFs of this serotype 16-specific gene cluster are responsible for the biosynthesis of serotype 16 GPL.**

Mycobacterial diseases, such as tuberculosis and infection due to nontuberculous mycobacteria (NTM), are still among the most serious infectious diseases in the world. The incidence is increasing because of the spread of drug-resistant mycobacteria and the human immunodeficiency virus (HIV) infection/AIDS epidemic (16, 17, 30). *Mycobacterium avium*-*Mycobacterium intracellulare* complex (MAC) is the most common among isolates of NTM and is distributed ubiquitously in the environment. MAC causes pulmonary and extrapulmonary diseases in both immunocompromised and immunocompetent hosts. It affects primarily patients with advanced HIV infection. MAC includes at least two mycobacterial species, *M. avium* and *M. intracellulare*, that cannot be differentiated on the basis of traditional physical and biochemical tests (1, 41).

The cell envelope of mycobacteria is a complex and unusual structure. The key feature of this structure is an extraordinarily high lipid concentration (6, 10). To better understand the pathogenesis of MAC infection, it is necessary to elucidate the molecular structure and biochemical features of the lipid components. Among MAC lipids, the glycopeptidolipid (GPL) is of particular importance, because it shows not only serotype-specific antigenicity but also immunomodulatory activities in the host immune responses (2, 9, 23). Structurally, GPLs are composed of two parts, a tetrapeptide-amino alcohol core and a variable oligosaccharide (OSE). C_{26}-C_{34} fatty acyl-D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol) is further linked with 6-deoxy talose (6-d-Tal) and 3,4-di-*O*-methyl rhamnose (3,4-di-*O*-Me-Rha) at D-*allo*-Thr and the terminal L-alaninol, respectively. This type of core GPL is found in all subspecies of MAC, shows a common antigenicity, and is further glycosylated at 6-d-Tal to form a serotype-specific OSE.

At present, 31 distinct serotype-specific GPLs have been identified serologically and chromatographically (9). Although the standard technique for differentiation of MAC subspecies

* Corresponding author. Mailing address: Department of Host Defense, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Phone: 81 6 6645 3746. Fax: 81 6 6645 3747. E-mail: fujiwara@med.osaka-cu.ac.jp.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 7 March 2008.

has been serotyping based on the OSE residue of its GPL, the complete structures of only 15 GPLs have been defined. In addition to the chemical structures of various GPLs, genes encoding the glycosylation pathways in the biosynthesis of GPL have been identified and characterized (12, 21, 31). Epidemiological studies have shown that MAC serotypes 4 and 8 are the most frequently isolated from patients, and MAC serotype 16 is one of the next most common groups (32, 40). It has been suggested that the serotypes of MAC isolates participate in their virulence (29), and thus, understanding of the structure-pathogenicity relationship of GPLs is necessary. In the present study, we demonstrate the complete OSE structure of the GPL derived from serotype 16 MAC (*M. intracellulare*), which has a unique terminal-acylated-amido sugar, and we characterized the serotype 16 GPL-specific gene cluster involved in the glycosylation of carbohydrates.

MATERIALS AND METHODS

Bacterial strains and preparation of GPL. *M. intracellulare* serotype 16 strain ATCC 13950^T (NF 115) was purchased from the American Type Culture Collection (Manassas, VA). Three clinical isolates of *M. intracellulare* serotype 16 (NF 116 and 117) and *M. avium* serotype 1 (NF 113) were maintained in The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association. The preparation of GPL was performed as described previously (18, 24, 26). Briefly, each strain of *M. intracellulare* serotype 16 was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) with 0.5% glycerol and 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment (Difco) at 37°C for 2 to 3 weeks. The heat-killed bacteria were sonicated, and crude lipids were extracted with chloroform-methanol (2:1, vol/vol). The extracted lipids were dried and hydrolyzed with 0.2 N sodium hydroxide in methanol at 37°C for 2 h. After neutralization with 6 N hydrochloric acid, alkaline-stable lipids were partitioned by a two-layer system of chloroform-methanol (2:1, vol/vol) and water. The organic phase was recovered, evaporated, and precipitated with acetone to remove any acetone-insoluble components containing phospholipids and glycolipids. The supernatant was collected by centrifugation, dried, and then treated with a Sep-Pak silica cartridge (Waters Corporation, Milford, MA) with washing (chloroform-methanol, 95:5, vol/vol) and elution (chloroform-methanol, 1:1, vol/vol) for partial purification. GPL was completely purified by preparative thin-layer chromatography (TLC) of Silica Gel G (20 by 20 cm, 250 μm; Uniplate; Analtech, Inc., Newark, DE). The TLC plate was repeatedly developed with chloroform-methanol-water (65:25:4 and 60:16:2, vol/vol/vol) until a single spot was obtained. After exposure of the TLC plate to iodine vapor, the GPL band was marked, and then, the silica gels were scraped off and the GPL was eluted with chloroform-methanol (2:1, vol/vol).

Preparation of OSE moiety. β elimination of GPL was performed with alkaline borohydride, and the OSE elongated from D-*allo*-Thr was released as described previously (18, 24). Briefly, the GPL was dissolved in ethanol, and an equal volume of 10 mg/ml sodium borohydride or borodeuteride in 0.5 N sodium hydroxide was added and then stirred at 60°C for 16 h. The reaction mixture was decationized with Dowex 50W-X8 beads (Dow Chemical Company, Midland, MI), collected, and evaporated under nitrogen to remove boric acid. The dried residue was partitioned in two layers of chloroform-methanol (2:1, vol/vol) and water. The upper aqueous phase was recovered and evaporated. In these processes, the serotype 16-specific OSE was purified as an oligoglycosyl alditol.

MALDI-TOF and MALDI-TOF/TOF MS analyses. The molecular species of the intact GPL was detected by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) with an Ultraflex II (Bruker Daltonics, Billerica, MA). The GPL was dissolved in chloroform-methanol (2:1, vol/vol) at a concentration of 1 mg/ml, and 1 μl was applied directly to the sample plate, and then 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1, vol/vol) was added as a matrix. The intact GPL was analyzed in the reflectron mode with an accelerating voltage operating in a positive mode of 20 kV (5). Then the fragment pattern of the OSE was analyzed with MALDI-TOF/TOF MS. The OSE was dissolved in ethanol-water (3:7, vol/vol), and the matrix was 10 mg/ml 2,5-dihydroxybenzoic acid in ethanol-water (3:7, vol/vol). The OSE and the matrix were applied to the sample plate according to the method for intact GPL and analyzed in the lift-lift mode.

GC and GC-MS analyses of carbohydrates and *N*-acylated short-chain fatty acid. To determine the glycosyl composition and linkage position, gas chromatography (GC) and GC-MS analyses of partially methylated alditol acetate derivatives were performed. Perdeuteromethylation was conducted by the modified procedure of Hakomori as described previously (18, 20). Briefly, the dried OSE was dissolved with a mixture of dimethyl sulfoxide and sodium hydroxide, and deuteromethyl iodide was added. The reaction mixture was stirred at room temperature for 15 min and then water and chloroform were added. The chloroform-containing perdeuteromethylated OSE layer was collected, washed with water two times, and then completely evaporated. Partially deuteromethylated alditol acetates were prepared from perdeuteromethylated OSE by hydrolysis with 2 N trifluoroacetic acid at 120°C for 2 h, reduction with 10 mg/ml sodium borodeuteride at 25°C for 2 h, and acetylation with acetic anhydride at 100°C for 1 h (8, 18, 25). To identify amino-linked fatty acids, acidic methanolysis of serotype 16 GPL was performed with 1.25 M hydrogen chloride in methanol (Sigma-Aldrich, St. Louis, MO) at 100°C for 90 min, and the fatty acid methyl esters were extracted with *n*-hexane under the cooled ice. GC was performed using a 5890 series II gas chromatograph (Hewlett Packard, Avondale, PA) equipped with a fused SPB-1 capillary column (30 m, 0.25-mm inner diameter; Supelco Inc., Bellefonte, PA). Helium was used for electron impact (EI)-MS and isobutane for chemical ionization (CI)-MS as a carrier gas. A JMS SX102A double-focusing mass spectrometer (JEOL, Tokyo, Japan) was connected to the gas chromatograph as a mass detector. The molecular separator and the ion source energy were 70 eV for EI and 30 eV for CI, and the accelerating voltage was 8 kV. The D and L configurations of Rha residues were determined by comparative GC-MS analysis of trimethylsilylated (*S*)-(+)-sec-butyl glycosides and (*R*)-(−)-sec-butyl glycosides prepared from an authentic standard L-Rha (19).

NMR analysis of GPL. The GPL was dissolved in chloroform-d ($CDCl_3$)-methanol-d_4 (CD_3OD) (2:1, vol/vol). To define the anomeric configurations of each glycosyl residue, ^1H and ^{13}C nuclear magnetic resonance (NMR) was employed. Both homonuclear correlation spectrometry (COSY) and ^1H-detected [^1H, ^{13}C] heteronuclear multiple-quantum correlation (HMQC) were recorded with a Bruker Avance-600 (Bruker BioSpin Corp., Billerica, MA), as described previously (9, 18, 24, 34).

Construction of *M. intracellulare* serotype 16 cosmid library. A cosmid library of *M. intracellulare* serotype 16 strain ATCC 13950^T was constructed as described previously (18). Bacterial cells were disrupted mechanically, and genomic DNA was extracted with phenol-chloroform and then precipitated with ethanol. Genomic DNA randomly sheared into 30- to 50-kb fragments in the extraction process was fractionated and electroeluted from agarose gels using a Takara Recochip (Takara, Kyoto, Japan). These DNA fragments were rendered blunt ended using T4 DNA polymerase and deoxynucleoside triphosphates and then were ligated to dephosphorylated arms of pYUB412 (XbaI-EcoRV and EcoRV-XbaI), which were the kind gifts of William R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY). The cosmid vector pYUB412 is an *Escherichia coli-Mycobacterium* shuttle vector with the *int-attP* sequence for integration into a mycobacterial chromosome, *oriE* for replication in *E. coli*, a hygromycin resistance gene, and an ampicillin resistance gene. After in vitro packaging using Gigapack III Gold extracts (Stratagene, La Jolla, CA), recombinant cosmids were introduced into *E. coli* STBL2 [F$^-$ *mcrA* Δ(*mcrBC-hsdRMS-mrr*) *recA1 endA1 lon gyrA96 thi supE44 relA1* l Δ(*lac-proAB*)] and stored at −80°C in 50% glycerol.

Isolation of cosmid clones carrying biosynthesis gene cluster of serotype 16 GPL and sequence analysis. Isolation of DNA from *E. coli* transductants was performed as described by Supply et al., with modifications (39). The colonies were picked, transferred to a 1.5-ml tube containing 50 μl of water, and then heated at 98°C for 5 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was used as the PCR template. PCR was used to isolate cosmid clones carrying the rhamnosyltransferase (*rtfA*) gene with primers rtfA-F (5′-TTTTGGAGCGACGAGTTCATC-3′) and rtfA-R (5′-GTGTAGTTGACCACGCCGAC-3′). *rtfA* encodes an enzyme responsible for the transfer of Rha to 6-d-Tal in OSE (14, 31). The insert of cosmid clone no. 253 was sequenced using a BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 310 gene analyzer (Applied Biosystems). The putative function of each open reading frame (ORF) was identified by similarity searches between the deduced amino acid sequences and known proteins using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and FramePlot (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) with the DNASIS computer program (Hitachi Software Engineering, Yokohama, Japan).

Transformation of *M. avium* serotype 1 strain with cosmid clone no. 253. An *M. avium* serotype 1 strain (NF113) was transformed with pYUB412-cosmid clone no. 253 by electroporation, and hygromycin-resistant colonies were iso-

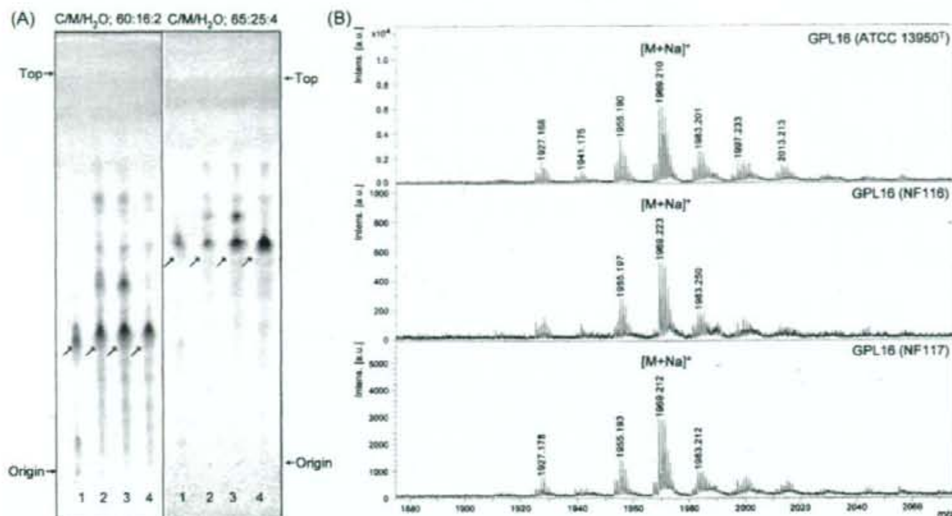

FIG. 1. TLC patterns and MALDI-TOF MS spectra of serotype 16 GPL. (A) Serotype 16 GPL purified from *M. intracellulare* ATCC 13950^T (NF 115) and the alkaline-stable lipids derived from ATCC 13950^T and two clinical isolates (NF 116 and 117) from left to right were developed on TLC plates with solvent systems of chloroform-methanol-water (65:25:4 and 60:16:2, vol/vol/vol). (B) The MALDI-TOF MS spectra were acquired using 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform-methanol (1:1, vol/vol) as a matrix, and the molecularly related ions were detected as $[M+Na]^+$ in positive mode. Intens., intensity; a.u., absorbance units.

lated. Alkaline-stable lipids were prepared, and productive GPLs were examined by TLC and MALDI-TOF MS analyses.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the NCBI GenBank database under accession no. AB355138.

RESULTS

Purification and molecular weight of intact GPL. Serotype 16 GPL from *M. intracellulare* ATCC 13950^T (NF 115) was detected as a spot by TLC, and the R_f values were 0.35 and 0.56 when developed with chloroform-methanol-water (60:16:2 and 65:25:4, vol/vol/vol, respectively). Two clinical isolates of *M. intracellulare*, NF 116 and 117, had serotype 16 GPLs that showed the same R_f values as the serotype 16 GPL derived from strain ATCC 13950^T. The serotype 16 GPL of *M. intracellulare* strain ATCC 13950^T was purified repeatedly by TLC and was shown as a single spot by TLC (Fig. 1A). The MALDI-TOF MS spectra of each serotype 16 GPL showed *m/z* 1969 for $[M+Na]^+$ as the main molecularly related ion in positive mode, with the homologous ions differing by 14 mass units at 1,955 and 1,983 (Fig. 1B). As a result, the main molecular weight of serotype 16 GPL was 1,946, which implied that it has a novel carbohydrate chain elongated from D-*allo*-Thr.

Carbohydrate composition of serotype 16 OSE. To determine the glycosyl compositions of serotype 16 OSE, alditol acetate derivatives of the serotype 16 GPL were analyzed by GC and GC-MS. The structurally defined serotype 4 GPL was used as a reference standard (9, 35). Comparison of the retention time and GC mass spectra (Fig. 2) with the alditol acetate derivatives of the serotype 16 GPL showed the presence of 3,4-di-*O*-Me-Rha, 4-*O*-Me-Rha, Rha, 6-d-Tal, and an unknown sugar residue (X1) in a ratio of approximately 1:1:2:1:1. The alditol acetate of X1 was eluted at a retention time of 29.3 min, greater than that of glucitol acetate on the SPB-1 column. The CI-MS spectrum of X1 was $[M+H]^+$ at *m/z* 520 as a parent ion and *m/z* 460 as a loss of 60 (acetate). The fragment ions of X1 sugar showed characteristic patterns in EI-MS. *m/z* 360 indicated the cleavage of C-3 and C-4, and *m/z* 300, 240, and 180 were fragmented with a loss of 60 (acetate). Similarly, *m/z* 374 indicated the cleavage of C-2 and C-3, and *m/z* 314 and 254 were fragmented with a loss of 60 (Fig. 3A and B). These results indicated that X1 was 3,6-dideoxy hexose (Hex). The odd molecular weight of X1, 519, and *m/z* 187, 127, and 59 implied the presence of one amido group esterified with a short-chain fatty acid, possibly. After methanolysis of serotype 16 GPL, the resultant fatty acid methyl esters were extracted carefully and analyzed by GC-MS. The EI-MS spectrum of a short-chain fatty acid methyl ester showed mass ions at *m/z* 176 ($[M]^+$), 145 ($[M-31]^+$), 117 ($[M-59]^+$), 99, 88, 85, and 59 (Fig. 3C) (33, 37). Taking the results together, X1 was structurally determined to be 3-2′-methyl-3′-hydroxy-4′-methoxy-pentanoyl-amido-3,6-dideoxy-Hex.

Glycosyl linkage and sequence of serotype 16 OSE. To determine the glycosyl linkage and sequence of the OSE, GC-MS of perdeuteromethylated alditol acetates and MALDI-TOF/TOF MS of the oligoglycosyl alditol from serotype 16 OSE

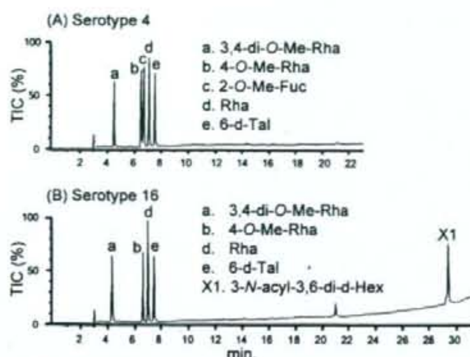

FIG. 2. Gas chromatograms of the alditol acetate derivatives from serotype 4 (A) and serotype 16 (B) GPLs. Total ion chromatograms (TIC) are shown. GC was performed on an SPB-1-fused silica column with a temperature program of 160°C for 2 min, followed by an increase of 4°C/min to 220°C, and holding at 220°C for 15 min. Comparison to the GC spectrum of serotype 4 GPL shows that serotype 16 GPL is composed of 3,4-di-*O*-Me-Rha, 4-*O*-Me-Rha, Rha, 6-d-Tal, and an unknown X1 sugar residue.

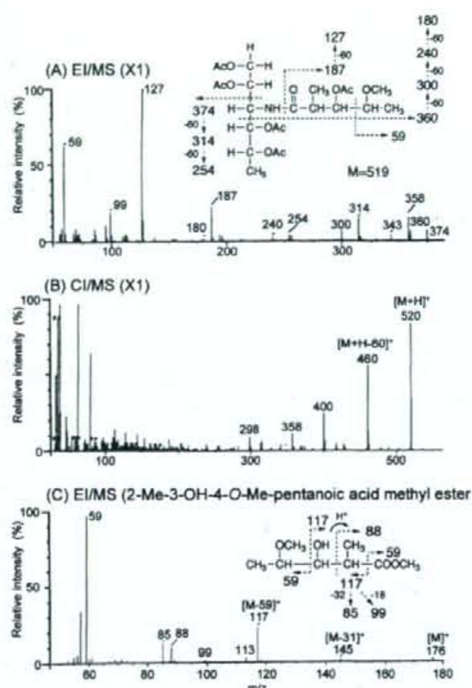

FIG. 3. EI-MS and CI-MS spectra of the alditol acetate derivative from X1 (A and B) and *N*-acylated-short-chain fatty acid methyl ester (C). The pattern of prominent fragment ions is illustrated. The CG column and condition were described in the legend for Fig. 2.

were performed. As shown in Fig. 4, the GC-MS spectra of perdeuteromethylated alditol acetates were assigned four major peaks, 1,3,4,5-tetra-*O*-deuteromethyl-2-*O*-acetyl-6-deoxytalitol (*m/z* 109, 132, 154, 167, and 214); 2,4-di-*O*-deuteromethyl-1,3,5-tri-*O*-acetyl-rhamnitol (*m/z* 121, 134, 205, 240, and 253); 2-*O*-deuteromethyl-4-*O*-methyl-1,3,5-tri-*O*-acetyl-rhamnitol (*m/z* 121, 131, 202, and 237); and 2,4-di-*O*-deuteromethyl-1,5-di-*O*-acetyl-3-2'-methyl-3'-*O*-deuteromethyl-4'methoxy-pentanoyl-deuteromethylamido-3,6-dideoxy-hexitol (*m/z* 121, 134, and 341). These results revealed that the 6-d-Tal residue was linked at C-2; Rha and 4-*O*-Me-Rha were linked at C-1 and C-3; and the nonreducing terminus, 3-2'-methyl-3'-hydroxy-4'-methoxy-pentanoyl-amido-3,6-dideoxy-Hex, was C-1 linked. The MALDI-TOF/TOF MS spectrum of the oligoglycosyl alditol from serotype 16 OSE afforded the expected molecular ions $[M+Na]^+$ at *m/z* 931, together with the characteristic mass increments in the series of glycosyloxonium ions formed on fragmentation at *m/z* 312, 472, 618, and 764 from the terminal sugar *N*-acyl-Hex to 6-d-Tal and at *m/z* 336, 482, and 642 from 6-d-Tal to *N*-acyl-Hex (Fig. 5). Rha residues were determined to be in the L absolute configuration by comparative GC-MS analyses of trimethylsilylated (*S*)-(+)-sec-butyl glycosides and (*R*)-(−)-sec-butylglycosides (see Fig. S1 in the supplemental material). Taken together, these results established the sequence and linkage arrangement 3-2'-methyl-3'-hydroxy-4'-methoxy-pentanoyl-amido-3,6-dideoxy-Hex-(1→3)-4-*O*-Me-Rha-(1→3)-L-Rha-(1→3)-L-Rha-(1→2)-6-d-Tal, exclusively.

NMR analysis of serotype 16 OSE. The ^{1}H NMR and ^{1}H-^{1}H COSY analyses of the serotype 16 GPL revealed six distinct anomeric protons with corresponding H1-H2 cross peaks in the low field region at δ4.93, 4.92, 4.92, 4.84, 4.65 ($J_{1,2}$ = 2 to 3 Hz, indicative of α-anomers) and 4.51 (a doublet, $J_{1,2}$ = 7.7 Hz, indicative of a β-hexosyl unit). When further analyzed by ^{1}H-detected [^{1}H, ^{13}C] two-dimensional HMQC, the anomeric protons resonating at δ4.93, 4.92, 4.92, 4.84, 4.65, and 4.51 have C-1s resonating at δ101.57, 95.73, 101.40, 102.56, 100.97, and 103.36, respectively (for a summary, see Table S1 in the supplemental material). The J_{CH} values for each of these protons were calculated to be 171, 170, 171, 170, 169, and 161 Hz by measurement of the inverse-detection nondecoupled two-dimensional HMQC (Fig. 6). These results established that the terminal amido-Hex was a β configuration and the others were α-anomers.

Cloning and sequence of serotype 16 GPL biosynthesis cluster. To isolate the serotype 16 GPL biosynthesis cluster, the genomic cosmid library of *M. intracellulare* serotype 16 strain ATCC 13950^T was constructed. Primers were designed to amplify the region corresponding to the *rtfA* gene. More than 300 cosmid clones were tested using colony PCR with *rtfA* primers, and the positive clones no. 51 and 253 were isolated from the *E. coli* transductants. PCR analysis revealed that clone no. 253 contained a *drrC* gene but that clone no. 51 did not. Thus, we used clone no. 253 for subsequent sequence analysis for the *gtfB-drrC* region. The 22.9-kb region of *M. intracellulare* sero-