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Evaluation of major membrane protein-I as a serodiagnostic tool of pauci-bacillary leprosy

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ABSTRACT

We have previously shown that the serodiagnosis using major membrane protein-II (MMP-II) is quite efficient in diagnosing leprosy. However, the detection rate of pauci-bacillary (PB) leprosy patients is still low. In this study, we examined the usefulness of major membrane protein-I (MMP-I) from *Mycobacterium leprae*. The MMP-I–based serodiagnosis did not show significantly high detection rate. However, when the mixture of MMP-I and MMP-II antigens was used, we detected 94.4% of multi-bacillary leprosy and 39.7% of PB patients. There were little correlation between the titers of anti–MMP-I antibodies (Abs) and that of anti-MMP-II Abs in PB patients' sera. Ten out of 46 MMP-II–negative PB leprosy patients were MMP-I positive, so that the detection rate of PB leprosy patient increased from 39.7% to 53.8% by taking either test positive strategy. We concluded that MMP-I can complement the MMP-II–based serodiagnosis of leprosy.

1. Introduction

Leprosy is chronic infectious disease caused by an infection with *Mycobacterium leprae* (*M. leprae*), and a significant number of new cases are still detected in 2011; for instance, 219,075 new cases were reported (WHO, 2012). Leprosy usually leads to peripheral nerve injury and systemic deformity (Job, 1989; Stoner, 1979), and the development of the deformity might be preventable, if the sufficient chemotherapy is initiated at an early stage of infection. Thus, early detection of leprosy is quite essential. As leprosy is mainly endemic in developing countries, easy and inexpensive diagnosis is strongly desired.

The diagnosis of leprosy is conducted based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected lesions. However, these methods have low sensitivity because *M. leprae bacilli* cannot be detected easily (Shepard and McRae, 1968). Although PCR-based molecular methods have been developed (Donoghue et al., 2001; Martinez et al., 2006; Phetsuksiri et al., 2006), it is not practical to perform PCR in resource-poor settings area. In this respect, serodiagnosis is a reasonable method to diagnose leprosy. Phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific, was discovered in 1981 (Hunter and Brennan, 1981). The PGL-I is currently accepted as the standard target antigen (Ag) for serodiagnosis of leprosy (Meeker et al., 1986; Schuring et al., 2006; Sekar et al., 1993). However, the method using PGL-I may be useful for the detection of multi-bacillary (MB) leprosy but is not sensitive enough for the detection of pauci-bacillary (PB) leprosy at least in some countries (Kai et al., 2008; Soebono and Klatser, 1991). In the previous study, we have focused on major membrane protein-II (MMP-II) from *M. leprae* (Maeda et al., 2007; Kai et al., 2008). MMP-II is one of the major proteins in the membrane fraction of *M. leprae*, and it induces immune response of host cells during infection (Maeda et al., 2005; Makino et al., 2005). We applied MMP-II as a serodiagnostic tool and found that the MMP-II–based serodiagnosis can increase the detection rate of PB leprosy patient. However, detection rate was still low at 39% (Maeda et al., 2007); thus, it is desirable to improve the sensitivity of the diagnostic tool.

In this study, we focused on major membrane protein-I (MMP-I) from *M. leprae*. MMP-I is 35-kDa major membrane protein expressed in *M. leprae*, which is identified as one of the most dominant Ags of *M. leprae* (Winter et al., 1995).

Although the function of MMP-I is still unknown, MMP-I may induce cell-mediated immune responses (unpublished observation) but has no homology with MMP-II. Therefore, MMP-I could be recognized by the different population of immune cells of leprosy patients and might be worth applying as a serodiagnostic Ag for the improvement of serodiagnosis. We purified recombinant MMP-I Ag using *Mycobacterium smegmatis* and evaluated its usefulness in the detection of both PB and MB leprosy patients.

2. Materials and methods

2.1. Study population

Sera were obtained with informed consent from healthy volunteers and leprosy patients in Japan. Frozen sera samples were used for the study. The samples studied comprised of MB ($n = 72$) and PB ($n = 78$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen. Classification of leprosy was performed by using the clinical criteria but was re-classified according to WHO

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recommendations (http://www.who.int/lep/classification/en/index.html) for study purposes. In Japan, children are obligated to get vaccination with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG); therefore, all healthy volunteers ($n = 78$) are likely to be BCG-vaccinated. Sera from healthy volunteers were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for the positivity. This study is approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.2. Purification of MMP-I and MMP-II

The MMP-I gene (ML0841) was cloned from the genome DNA of *M. leprae*, using primers: 5′-GAGGATCCACGTCGGCTCAGAATGAGTC-3′ and 5′-ATACTAGTTCACTTGTACTCATGGAACT-3′. The amplified gene was expressed in *M. smegmatis* using pMV261 expression vector. The recombinant protein was His6-tagged and purified with Cu^{2+} resin (ABT Agarose Bead Technologies, Tampa, FL, USA). The protein obtained was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels, then the gel was stained with Instant Blue (Expedeon protein solution, San Diego, CA, USA), and a single band of MMP-I protein was observed. The MMP-II gene (ML2038c) was expressed and purified as previously described (Maeda et al., 2007).

2.3. ELISA

The ELISA for the detection of anti-MMP-I antibodies (Abs) was performed as described previously with several modifications (Maeda et al., 2007). Briefly, 96-well plates (Nunc Maxisorp, Thermo Fischer Scientific Inc., Waltham, MA, USA) were coated overnight with MMP-I Ag at a concentration of 1 μg/mL, MMP-II Ag at 2 μg/mL, or the mixture of MMP-I (1 μg/mL) and MMP-II (2 μg/mL) Ags. All Ags were diluted in 0.1 mol/L carbonate buffer (pH 9.5). After blocking with 10% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS), the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). The optimal concentration of both Ags was determined in advance. Human sera diluted 100-fold were added and incubated at room temperature for 2 hours. After washing with PBS-T, biotinylated anti-human IgG Ab (Vector Laboratories, Burlingame, CA, USA) was added at a concentration of 0.5 μg/mL and incubated for 1 hour. Then, the plates were incubated with reagents from a Vecstain ABC kit (Vector Laboratories) for 30 min. These reagents include avidin and biotinylated horse-radish peroxidase, and this enzyme binds to biotinylated anti-human IgG Ab via avidin. After further washing with PBS-T, a substrate solution consisting of 0.2 mg/mL of 2,2′-Azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) and 0.02% H_2O_2 in 0.1 mol/L citrate buffer was added until a blue color developed, and the reaction was stopped by adding 2 N H_2SO_4. Optical density (OD) was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.360. The volume of all solutions used in the 96-well plate was 50 μL/well.

2.4. Statisitical analyses

The data were analyzed using MEDCALC software (MedCalc, Ostend, Belgium). A receiver operating characteristic (ROC) curve was drawn to calculate the cut-off levels using the OD values of MB leprosy patients' sera and healthy controls. The McNemar test was applied to determine the *P* value. When the number of inconsistent pairs was less than or equal to 25, the calculation of 2-sided *P* value was done based on the cumulative binomial distribution. The *P* value of <0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the 2 tests.

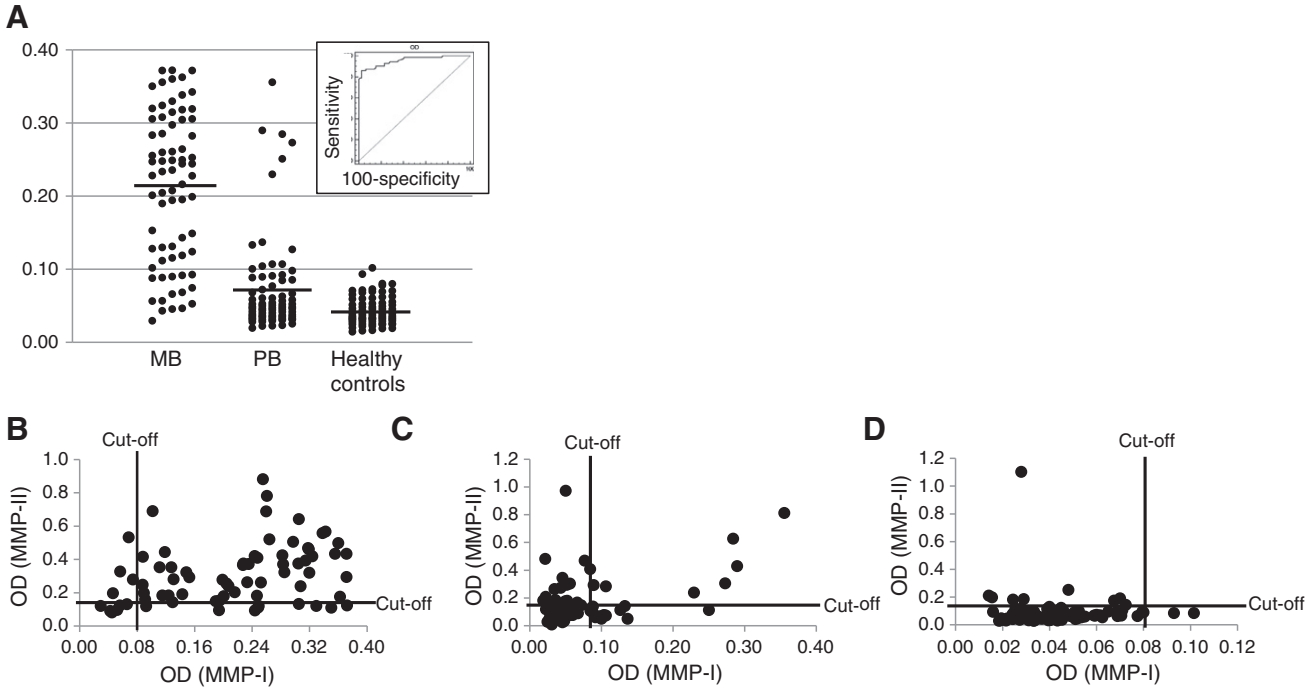

Fig. 1. OD values of each sample were determined by MMP-I–based serodiagnosis and MMP-II–based serodiagnosis. (A) Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I–based ELISA. OD value (wave length: 405 nm) of each sample was plotted. The thick horizontal lines show the average of OD in each group. (Inset) ROC curve analysis of MMP-I–based ELISA. The cut-off value was determined as 0.080, and the area under the ROC curve was 0.952. (B–D) The results of MMP-I–based ELISA and MMP-II–based ELISA were plotted. Sample sera from MB leprosy patients (B), PB leprosy patients (C), and healthy controls (D) were subjected to MMP-I–based or MMP-II–based ELISAs. The x-axis shows the OD value of MMP-I–based ELISA, and the y-axis shows that of MMP-II–based ELISA. Thick lines show the cut-off value of each analysis (MMP-I, 0.080; MMP-II, 0.13).