J. Biochem. 2009;146(5)659–665
doi:10.1093/jb/mvp113

Mycolyltransferase from *Mycobacterium leprae* Excludes Mycolate-containing Glycolipid Substrates

Hitomi Nakao[1,2,*], Isamu Matsunaga[1,2,*,†], Daisuke Morita[1,2], Takako Aboshi[3], Toshiyuki Harada[3], Yoshiaki Nakagawa[3], Naoki Mori[3] and Masahiko Sugita[1,2]

[1]Laboratory of Cell Regulation, Institute for Virus Research, Kyoto University; [2]Laboratory of Cell Regulation and Molecular Network, Graduate School of Biostudies; and [3]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Received June 23, 2009; accepted July 3, 2009; published online July 23, 2009

Trehalose dimycolate (TDM) is a major surface-exposed mycolyl glycolipid that contributes to the hydrophobic cell wall architecture of mycobacteria. Nevertheless, because of its potent adjuvant functions, pathogenic mycobacteria appear to have evolved an evasive maneuver to down-regulate TDM expression within the host. We have shown previously that *Mycobacterium tuberculosis* (M.tb) and *Mycobacterium avium* (M.av), replace TDM with glucose monomycolate (GMM) by borrowing host-derived glucose as an alternative substrate for the FbpA mycolyltransferase. *Mycobacterium leprae* (M.le), the causative microorganism of human leprosy, is also known to down-regulate TDM expression in infected tissues, but the function of its mycolyltransferases has been poorly analysed. We found that, unlike M.tb and M.av FbpA enzymes, M.av FbpA was unexpectedly inefficient in transferring α-branched mycolates, resulting in impaired production of both TDM and GMM. Molecular modelling and mutational analysis indicated that a bulky side chain of leucine at position 130 of M.le FbpA obstructed the intramolecular tunnel that was proposed to accommodate the α-branch portion of the substrates. Notably, even after a highly reductive evolution, M.le FbpA remained functional in terms of transferring unbranched acyl chains, suggesting a role that is distinct from that as a mycolyltransferase.

Key words: glycolipid, glucose monomycolate, *Mycobacterium leprae*, mycolyltransferase, trehalose dimycolate.

Abbreviations: Fbp, fibronectin-binding protein; GMM, glucose monomycolate; M.av, *Mycobacterium avium*; M.le, *Mycobacterium leprae*; M.tb, *Mycobacterium tuberculosis*; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

Mycobacteria are unique in their highly lipid-rich cell wall that is critical not simply for their acid-fast properties but also for their survival and replication. The cell wall contains mycolic acids, a family of α-branched, β-hydroxy long-chain fatty acids, which are densely aligned in covalent association with the underlying arabinogalactan sugar layer or exist as free molecules complexed to either trehalose or glucose. The arabinogalactan-bound mycolic acids are proposed to extend outwards and interact closely with carbon chains of the surface-exposed glycolipids, thereby forming the hydrophobic cell wall architecture that is essential for long-term survival of pathogenic mycobacteria within host cells (*1*).

Trehalose-6,6′-dimycolate (TDM) comprises a major surface-exposed mycolyl glycolipid that can be readily synthesized when mycobacteria are cultured in artificial media, and therefore, its biological activities as well as its relevance to pathogenesis have been studied extensively over the past two decades. A single dose of TDM can induce granuloma formation *in vivo* in animal tissues, and its outstanding ability to stimulate host innate immune cells, such as macrophages and dendritic cells, has been fully documented (*2*). Further, we recently reported TDM-elicited eosinophilic responses in mycobacteria-infected guinea pigs (*3*). All of these immunostimulatory or adjuvant functions mediated by TDM could potentially jeopardize the microbes by allowing the host to efficiently monitor and control infection. The generally accepted picture of the cell wall structure of mycobacteria, highlighting abundant TDM expression at the outermost layer, has been drawn, based primarily on biochemical analysis of the microbes cultivated in artificial media, but adaptive changes that minimize TDM functions may occur after pathogenic mycobacteria infect into the host.

Indeed, we have recently shown that a switch of glycolipid biosynthesis from TDM to another surface-exposed mycolyl glycolipid, glucose-6-monomycolate (GMM), occurs in *Mycobacterium tuberculosis* (M.tb) and *Mycobacterium avium* (M.av) upon exposure to host-derived glucose (*4*). Previous studies have

*These two authors contributed equally to this work.
†To whom correspondence should be addressed. Tel: +81-75-751-4047, Fax: +81-75-752-3232,
E-mail: i_matsun@virus.kyoto-u.ac.jp
Correspondence may also be addressed to Masahiko Sugita. Tel: +81-75-751-4028, Fax: +81-75-752-3232,
E-mail: msugita@virus.kyoto-u.ac.jp

established that the final step of TDM biosynthesis is catalysed by the mycolyltransferase activity of the fibronectin-binding protein (Fbp), using its biosynthetic precursor, trehalose-6-monomycolate (TMM) as a substrate (*5*). However, at the physiological concentration of glucose present in mammalian hosts, TDM down-regulation and concomitant GMM up-regulation occur within hours as a result of competitive acceptor substrate selection of TMM and glucose by the Fbp enzyme (*4*). Given that GMM is much less potent than TDM in stimulating innate immune cells, the swift switch from TDM to GMM biosynthesis by utilizing the pre-existing Fbp enzyme and the host-derived glucose could function as an efficient evasive maneuver for pathogenic mycobacteria (*4*).

Leprosy is an ancient disease caused by *Mycobacterium leprae* (M.le) infection, but remains an important health problem worldwide. Unlike M.tb and M.av, M.le is not cultivable in artificial media, and therefore, its lipid chemistry and biology have not been studied so extensively as for the other pathogenic mycobacteria species. Previous studies have suggested that only a trace amount of TDM is produced by M.le grown in infected tissues while production of its biosynthetic precursor, TMM, is readily detectable (*6*). Nevertheless, the M.tb and the M.le genomes share all the four Fbp genes (*fbpA*, *fbpB*, *fbpC* and *fbpD*), and the deduced amino acid sequences indicate that products of M.le *fbpA*, *fbpB* and *fbpC*, designated FbpA, FbpB and FbpC, respectively, contain a catalytic triad that is essential for the mycolyltransferase activity, as found in other serine esterases (*7*). Therefore, it was initially hypothesized that M.le should have evolved an efficient strategy for TDM down-regulation that might be similar to maneuvers employed by M.tb and M.av. Surprisingly, however, we provide evidence that the M.le-derived FbpA protein excludes mycolate-containing glycolipid substrates, resulting in profound down-regulation of both TDM and GMM. This 'intrinsic' mechanism of TDM down-regulation has not been observed for other pathogenic mycobacteria species tested so far, and may uniquely support survival of M.le within the host.

MATERIALS AND METHODS

Chemical Reagents and Bacteria—Chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. *Mycobacterium avium* ATCC 35767 (serovar 4) was obtained from American Type Culture Collection (Manassas, VA, USA). The bacteria were grown in Middlebrook 7H9 media (BD, Franklin Lakes, NJ, USA) supplemented with the albumin-dextrose-catalase enrichment (BD) and 0.05% Tween 80. *Corynebacterium matruchotii* JCM 9386 was obtained from Japan Collection of Microorganisms (Tsukuba, Japan), and maintained on a plate of brain-heart-infusion agar media (BD). For isolation of TMM from *C. matruchotii*, the bacteria were grown in brain-heart-infusion liquid media containing 5% trehalose.

Preparation of Recombinant Enzymes—The recombinant M.av FbpA was prepared as described previously (*4*). The gene that encoded the mature M.le FbpA protein lacking the signal sequence was amplified by PCR, using genomic DNA of the M.le Thai-53 strain (*8*) as a template and a specific primer set as follows: 5′-ggaattcca tatgttctcccggccgggattgcc-3′ (sense primer) and 5′-cccaagct tagcacccggggtagccccta-3′ (antisense primer). The amplified PCR product was digested with NdeI and HindIII, and ligated to a NdeI–HindIII-digested pET-21c plasmid vector (Merck Japan, Tokyo, Japan). The nucleotide sequence of the cloned M.le *fbpA* was identical to that reported elsewhere (http://genolist.pasteur.fr/Leproma/). To optimize the codon usage for efficient expression in *Eschericia coli*, PCR was carried out with the cloned M.le *fbpA* gene as a template and a primer set as follows: 5′-cgcatatgttctctcgtccgggtctgccggttgagtacctgcaa-3′ and 5′-gccccggggtagcacccaggtactgctgcaggtccggtttcatg-3′ (sense and antisense primers, respectively, in which mutated nucleotides are underlined). The PCR product was digested with NdeI and SmaI, and exchanged for the corresponding fragment of the cloned M.le *fbpA* gene in pET-21c.

Site-directed mutagenesis was performed by PCR with the PrimeStar HS DNA polymerase (Takara Co. Ltd, Tokyo, Japan), using the codon-modified plasmids as a template. The primer sets used was as follows: 5′-cgg ttcttcggccctgacgctggcgatctaccaccc-3′ and 5′-ggccgaagaaccg gccatcgaaagaccgaccgccgc-3′ (for M.le *fbpA*); 5′-cggcctgtcgg cgctgatcctggccgcctaccac-3′ and 5′-ccgacaggccggccatcgac aggccgacgacacc-3′ (for M.av *fbpA*), in which mutated nucleotides are underlined. The cycling conditions for PCR amplification were as follows: 95°C, 1 min, followed by 10 cycles of 95°C, 15 s and 72°C, 10 min, and a final extension step of 72°C, 10 min. The amplified PCR products were digested with DpnI, and used for transformation of *E. coli*. Introduction of the mutation was confirmed by DNA sequencing.

Escherichia coli BL21 (DE3) was transformed with each of the plasmids, and the His-tagged recombinant FbpA proteins were obtained as described (*4*) with slight modifications. The protein expression was induced with 0.1 mM IPTG at 25°C for 20 h. The cells were then harvested and disrupted by sonication in ice-cold 20 mM Tris–HCl (pH 7.9) buffer containing 0.5 M NaCl and 60 mM imidazole (sonication buffer). The sonicates were centrifuged at 6,000*g* for 30 min at 4°C to remove insoluble materials, and the supernatants were applied onto a Ni-resin column equilibrated with the sonication buffer. After washing the column with the sonication buffer, the recombinant proteins were eluted with the 20 mM Tris–HCl (pH 7.9) containing 0.5 M NaCl and 0.5 M imidazole. The eluates were concentrated and dialyzed overnight at 4°C against 50 mM sodium phosphate buffer (pH 7.5) containing 10% glycerol at 4°C. Purity and quantity of the enzymes were assessed by SDS–PAGE and Coomassie staining.

Preparation of Substrates—The M.av serovar 4-derived long-chain TMM was purified as described (*4*). For purification of the *C. matruchotii*-derived short-chain TMM, the cultured cells were harvested, and lipids were extracted with chloroform/methanol (C/M, 1 : 1, v/v), followed by fractionation by TLC with a solvent system of C/M/water (65 : 25 : 4, v/v/v). The TMM fraction was then extracted with C/M (1:1, v/v) from the silica gels.

The identity of the purified TMM preparations was confirmed by mass spectrometry.

Trehalose-6-monolaurate (TML) was synthesized by modification of a method of Raku *et al.* (*9*). The reaction mixture (dimethylformamide/water, 97 : 3, v/v) containing 160 mM α-trehalose (Wako Pure Chemicals Co. Ltd., Osaka, Japan), 450 mM vinyl laurate (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) and 31 mg/ml Bioprase OP (Nagase Chemtex Co. Ltd, Osaka, Japan) was incubated at 35°C for 7 days, and then extracted with *n*-hexane to remove unreacted vinyl laurate. The dimethylformamide phase was applied onto a C18 reverse-phase column (Presep C18 ODS, Wako Pure Chemicals) that was equilibrated with water. Subsequently, the column was washed with water, and the TML-containing fraction was eluted with acetonitrile/water (50 : 50, v/v). After evaporation, the fraction was dissolved in methanol and applied onto a silica gel G TLC plate (Analtech, Newark, NJ, USA). The plate was developed with a solvent system of C/M/acetone/acetic acid (50 : 30 : 20 : 1, v/v/v/v), and TML was extracted from the gel. Its identity was confirmed by mass spectrometry and ^{1}H NMR.

Enzyme Assays—FbpA enzyme assays were performed as described (*4*) with slight modifications. Each of the lipidic substrates (63 μM long-chain TMM, 130 μM short-chain TMM and 1.9 mM TML) was prepared in 20 mM sodium phosphate buffer (pH 7.5) (reaction buffer) unless otherwise indicated. In experiments monitoring GMM production, the reaction mixtures also contained 4% D-glucose (w/v). The reaction was started by the addition of either M.le-derived or M.av-derived FbpA enzymes (1.5 nmol for long-chain TMM, 160 pmol for short-chain TMM, and 7.8 pmol for TML) with a total volume of 200 ml per tube, and after 20 min of incubation at 37°C, the reaction was stopped by the addition of 3 ml of C/M (2 : 1) and 0.3 ml of distilled water as well as *n*-tetradecanol (20 μg per sample) that served as an indicator for extraction efficiency. The lipids were extracted by the method of Kremer *et al.* (*10*) and analysed by silica gel TLC with a solvent of either C/M/acetone/acetic acid (90 : 10 : 10 : 1) (for detection of long-chain mycolyl glycolipids) or C/M/acetone/acetic acid (80 : 15 : 10 : 1) (for detection of lauryl trehalose and short-chain mycolyl glycolipids). The lipids on the TLC plates were visualized by spraying 50% sulphuric acid and baking. The amounts of each compound were calculated, based on the intensity of spots of serially diluted *n*-tetradecanol. The molecular identity of the products was confirmed by mass spectrometry, using an electrospray-ion trap-time of flight mass spectrometer (Shimadzu LCMS-IT-TOF, Shimadzu Co. Ltd, Kyoto, Japan) as described (*11*).

Kinetic Analysis of Enzyme Reactions—Enzyme assays were carried out with wild-type and mutant FbpA enzymes, using four different concentrations of the *C. matruchotii*-derived short-chain TMM as a substrate. The lipids were extracted and separated on TLC plates, followed by densitometrical determination of the amount of the products, using serially diluted *n*-tetradecanol as a reference. Data were collected from three independent experiments, and kinetic analysis was performed by Hanes–Woolf plotting.

Molecular Modelling of the FbpA S130L Mutant—Molecular modelling of the M.tb-derived FbpA S130L mutant protein was performed, using the homology modelling software PDFAMS (Protein Discovery Full Automatic Modeling System; In-Silico Sciences, Inc., Tokyo, Japan) as described (*12*, *13*). Briefly, the primary sequence and the molecular model of the M.tb FbpA protein were obtained from the Protein Data Bank (1SFR). The serine residue at position 130 was mutated into leucine, and the obtained 3D structure was optimized by the simulated annealing method. Subsequently, the molecular model was subjected to energy minimization, using the SYBYL software (version 7.3; Tripos Inc., St Louis, MO, USA). The surface of the channel that was proposed to accommodate the α-branch portion of the substrates was depicted by utilizing the MOLCAD module of SYBYL.

RESULTS

M.le FbpA Exhibits Reduced Mycolyltransferase Activity—Recombinant FbpA enzymes derived from M.le and M.av were obtained and tested for their mycolyltransferase activity in an *in vitro* enzyme assay, using mycobacteria-derived natural TMM as a substrate. As we have shown previously (*4*), an efficient transfer of the mycolyl acyl group from one TMM substrate (donor) to the other TMM substrate (acceptor) occurred in the presence of the M.av FbpA protein, evidenced by generation of the reaction product, TDM (Fig. 1, lane 1).

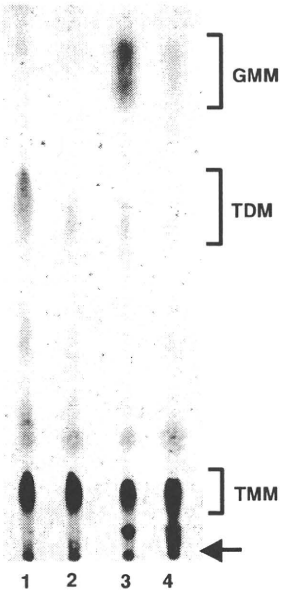

Fig. 1. **Reduced mycolyltransferase activity of M.le FbpA.** Enzymatic reactions with the long-chain TMM as a substrate were performed at 37°C for 20 min at conditions indicated below, and the lipids were extracted from the reaction mixtures, followed by analysis on a TLC plate. Lane 1, M.av FbpA and TMM; lane 2, M.le FbpA and TMM; lane 3, M.av FbpA, TMM and glucose; lane 4, M.le FbpA, TMM and glucose. The TLC origin is indicated with an arrow.

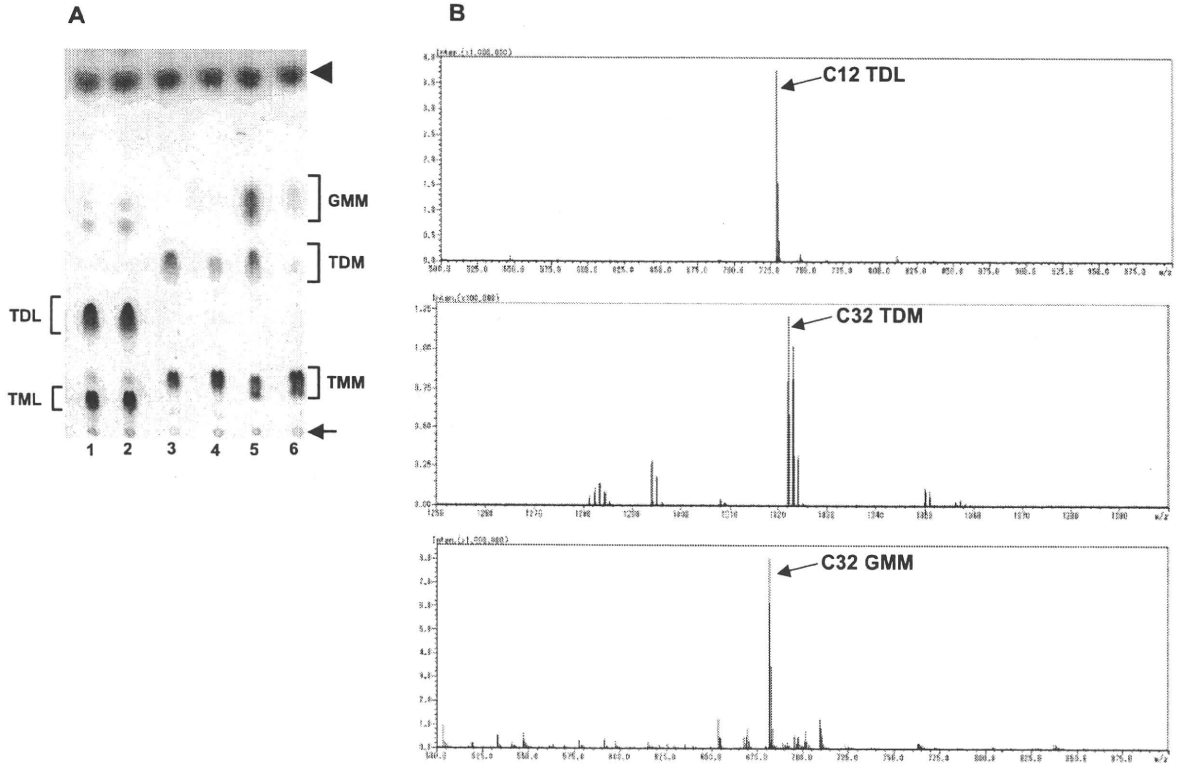

Fig. 2. **Efficient utilization of unbranched, but not α-branched, substrates by M.le FbpA.** (A) Enzymatic reactions were performed as in Fig. 1, using either TML or the short-chain TMM as a substrate. Lane 1, M.av FbpA and TML; lane 2, M.le FbpA and TML; lane 3, M.av FbpA and TMM; lane 4, M.le FbpA and TMM; lane 5, M.av FbpA, TMM and glucose; lane 6, M.le FbpA, TMM and glucose. The position of the extraction efficiency indicator, *n*-tetradecanol, is indicated with an arrowhead. Note that equivalent amounts of *n*-tetradecanol were visualized throughout the lanes. (B) The reaction products, TDL (top panel), *C. matruchotii* TDM (middle panel) and GMM (bottom panel), were extracted from the TLC plates and analysed by mass spectrometry. The major ions of m/z 729.4 (top panel), m/z 1322.0 (middle panel) and m/z 681.5 (bottom panel) were indicated with arrows that represent sodium adducts of C_{12} TDM, TDM with two molecules of C_{32} mycolate, and C_{32} mycolate-containing GMM, respectively.

In sharp contrast, only a tiny amount of TDM was detected when an equivalent amount of the M.le FbpA protein was used (lane 2). The mycolyltransferase activity of the M.av and M.le FbpA proteins was also assessed in parallel, using glucose as an alternative acceptor substrate. Similar to TDM, much more efficient generation of the reaction product, GMM, was observed for M.av FbpA (lane 3), and only a trace of GMM was produced by M.le FbpA (lane 4). Therefore, these results detected apparently reduced mycolyltransferase activity for M.le FbpA.

M.le FbpA Retains the Ability to Transfer Unbranched, but not α-branched, Fatty Acids—The mycobacteria-derived TMM molecules used as a substrate for the enzyme reactions above were those containing α-branched, long-chain (mainly C_{85}) fatty acids. To gain insight into the molecular basis for the decreased mycolyltransferase activity exhibited by M.le FbpA, similar *in vitro* enzyme reaction experiments were performed, using two monoacyl trehalose compounds as model substrates; namely, synthesized TML with a C_{12} unbranched acyl chain, and *C. matruchotii*-derived TMM with α-branched, short-chain (mainly C_{32}) fatty acids. We found that both M.av and M.le FbpA proteins were capable of transferring the C_{12} unbranched acyl chain from the donor TML molecule to the acceptor TML molecule efficiently, resulting in generation of a comparable amount of trehalose-6,6′-dilaurate (TDL) by both enzymes (Fig. 2A, lanes 1 and 2). The molecular identity of the product as TDL was confirmed by mass spectrometry, in which the mass numbers of given ions were matched with those for sodium adducts of C_{12} TDL (Fig. 2B, top panel). On the other hand, the *C. matruchotii*-derived α-branched, short-chain TMM was not utilized efficiently as a donor substrate for M.le FbpA, evidenced by reduced TDM and GMM production as compared with M.av FbpA (Fig. 2A, lane 3 versus lane 4 for TDM production, and lane 5 versus lane 6 for GMM production). The molecular identity of the products as TDM and GMM was confirmed by mass spectrometry (Fig. 2B, middle and bottom panels, respectively). From these observations, we hypothesized that, unlike monoacyl trehalose compounds with an unbranched fatty acid, those containing the fatty acyl branching of mycolic acids could

not gain easy access to the donor substrate-binding site of the M.le FbpA protein.

Leucine at Position 130 of M.le FbpA Limits Access of Mycolate-containing Glycolipid Substrates to their Binding Site—Crystallographic studies of M.tb-derived mycolyltransferases detected a tunnel extending through the core of the protein that could accommodate the α-branch portion of mycolates while their meromycolate chain fitted in a long cleft formed on the surface of the enzyme (*14–16*). The fact that even the α-branch of the short-chain TMM could not be efficiently utilized by the M.le FbpA enzyme might indicate a failure of the α-branch to penetrate deeply into the tunnel due to blockade at or near its opening. We therefore looked closely at the α4 helix (Fig. 3A, boxed area) that contributed to the formation of the tunnel in the proximity of the position 126 serine residue (indicated with an asterisk) of the catalytic triad. Alignment of the amino acid sequences of M.le-, M.av- and M.tb-derived FbpA proteins shows that the α4 helix sequence is highly conserved, but the position 130 serine residue found in both M.av and M.tb is replaced by leucine in M. le (Fig. 3, indicated with an arrowhead).

In the crystal structure of the M.tb FbpA protein reported previously, the side chain of the position 130 serine residue (Ser130) protrudes into the lumen of the tunnel (demarked with dotted lines), generating a kinky channel, but there is still a plenty of open space that allows smooth accommodation of the α-branch portion of the substrates (Fig. 3B, left panel). On the other hand, molecular modeling after serine-to-leucine mutation was introduced at position 130 revealed that the bulky side chain of the leucine residue protruded further, resulting in total obstruction of the tunnel (Fig. 3B, right panel). Thus, we suspected that the impaired utilization of α-branched fatty acid-containing substrates by M.le FbpA might be accounted for by the presence of the leucine residue at position 130.

To test this possibility directly, recombinant M.le- and M.av-derived FbpA proteins with a single amino acid mutation at position 130 were generated and assessed for their acyl-chain transfer activity. The mutant M.av FbpA protein in which the serine residue was replaced with leucine (M.av FbpA S130L) exhibited a reduced mycolyltransferase activity (Fig. 4A, lane 4) as compared with the wild-type M.av FbpA protein (lane 3) while maintaining the ability to transfer the C_{12} unbranched acyl chain (lanes 1 and 2). In sharp contrast, the mutant M.le FbpA protein in which the leucine residue was replaced with serine (M.le FbpA L130S) exhibited an enhanced mycolyltransferase activity (Fig. 4B, lane 4) as compared with the wild-type M.le FbpA

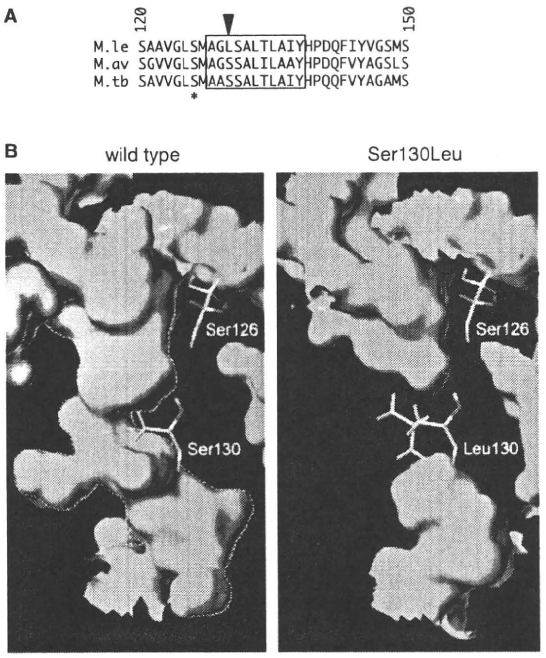

Fig. 3. **A critical amino acid residue at position 130 of FbpA proteins that regulates interaction with α-branched substrates.** (A) Partial amino acid sequences of M.le-, M.av- and M.tb-derived FbpA proteins are aligned. Residues from position 120 to position 150 are shown, and the position 130 is indicated with an arrowhead. The boxed area indicates amino acid sequences of the α4 helix. The serine residue of the catalytic triad located at position 126 is indicated with an asterisk. (B) Structure of the M.tb wild type FbpA (left panel) and molecular modeling of its Ser130Leu mutant (right panel) are shown. The surface of open space is depicted, and the intramolecular channel that accommodates the α-branch portion of the substrates is demarcated with dotted lines. Note that the tunnel totally collapsed after the introduction of the mutation.

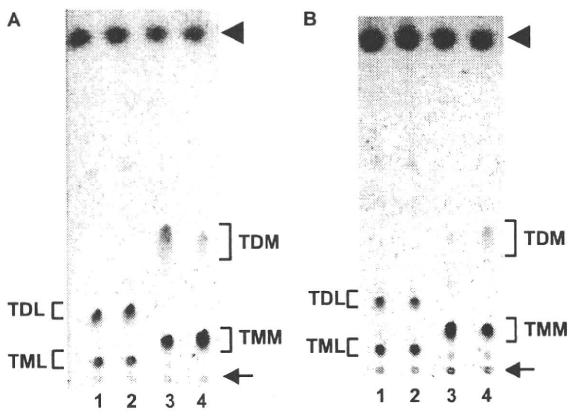

Fig. 4. **Effects of amino acid substitution at position 130 of FbpA proteins on mycolyltransferase activity.** (A) Enzymatic reactions were performed with either the wild-type M.av FbpA or its mutant (M.av FbpA S130L) as in Fig. 1 except for the use of TML at 0.3 mM. Lane 1, wild-type M.av FbpA and TML; lane 2, M.av FbpA S130L and TML; lane 3, wild-type M.av FbpA and short-chain TMM; lane 4, M.av FbpA S130L and short-chain TMM. (B) Similar enzymatic reactions were performed with either the wild-type M.le FbpA or its mutant (M.le FbpA L130S). Lane 1, wild-type M.le FbpA and TML; lane 2, M.le FbpA L130S and TML; lane 3, wild-type M.le FbpA and short-chain TMM; lane 4, M.le FbpA L130S and short-chain TMM. The TLC origin and the position of *n*-tetradecanol are indicated with arrows and arrowheads, respectively.

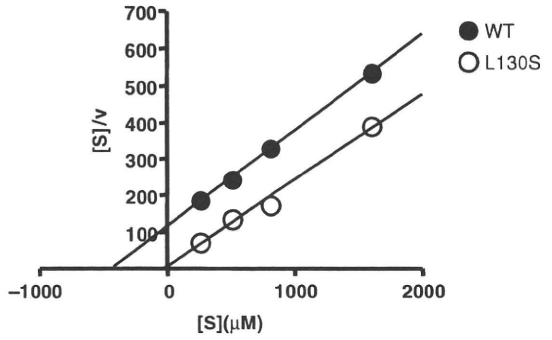

Fig. 5. **Distinct enzyme kinetics for M.le wild-type and mutant FbpA proteins.** Enzyme assays were performed with either wild-type or mutant FbpA proteins, using different concentrations of the short-chain TMM as a substrate. The lipids were extracted, and the amount of the product was densitometrically determined on TLC plates. Hanes–Woolf plots are shown for the wild-type (closed circles) and the mutant (open circles) FbpA enzymes.

protein (lane 3), while the ability to transfer the unbranched acyl chain was unchanged (lanes 1 and 2).

To quantitatively assess the enzyme activity of the M.le FbpA wild-type and mutant proteins, enzyme assays were carried out in the presence of varying amounts of the short-chain TMM substrate, and the amount of the product, TDM, generated at each concentration was determined, followed by kinetic analysis of the reactions using Hanes-Woolf plotting. As shown in Fig. 5, separate, but almost parallel, linearity was obtained for the wild-type and mutant enzymes, indicating a sharp decrease in the apparent K_m value after the amino acid substitution while the V_{max} values remained almost unaffected. The calculated K_m values were 459 μM for the wild-type and 43 μM for the mutant, and the V_{max} values were 3.83 nmol/min/nmol enzyme for the wild-type and 4.27 nmol/min/nmol enzyme for the mutant, underscoring an increased affinity of the TMM substrate to the mutant enzyme. Therefore, the amino acid substitution experiments detected a critical role for the leucine residue at position 130 in excluding α-branched mycolates, which could account for the reduced mycolyltransferase activity of the M.le FbpA enzyme.

DISCUSSION

TDM is produced abundantly by virtually all cultivable mycobacterial pathogens, and its relevance to pathogenesis has been proposed (*1*, *2*). Nevertheless, given its potent adjuvant effects on a wide variety of host immune cells, it would be reasonable to speculate that, upon infection into the host, pathogenic mycobacteria may down-regulate the TDM expression to allow for their escape from the host immune system. In this respect, the mycolyltransferase-mediated switch of glycolipid biosynthesis from TDM to GMM by borrowing host-derived glucose could function as an exquisite maneuver that pathogenic mycobacteria, such as M.tb and M.av, are able to employ (*4*). The cell wall lipid components of M.le remain to be fully characterized due to the highly limited sources of the microbe, but it has been suggested that the expression of TDM could be far below the level that would be expected for other mycobacteria (*6*), implicating a highly efficient mechanism for TDM down-regulation. Therefore, it is important to determine how TDM biosynthesis is regulated in M.le in order to understand the unique immunopathological features of leprosy.

The function of M.le-derived mycolyltransferases has not been specifically addressed previously. By utilizing recombinant proteins and an array of substrates in *in vitro* enzyme assays, the present study has disclosed for the first time unexpected features of the M.le-derived FbpA enzyme that are unique to M.le, but not to other pathogenic mycobcteria. Unlike the M.av FbpA protein capable of catalysing TDM synthesis from its precursor, TMM, the M.le enzyme was inefficient as a mycolyltransferase to generate TDM while maintaining its catalytic activity to transfer an unbranched acyl chain (Figs 1 and 2). Further, the site-directed mutagenesis studies, identifying the amino acid residue at position 130 as a key determinant for the accessibility of the α-branch portion of mycolates, have provided a molecular basis for the reduced mycolyltransferase activity of the M.le FbpA enzyme (Figs 4 and 5). Thus, these observations underscore 'intrinsic' defects of TDM production in M.le, which contrasted sharply with the 'extrinsic' pathway for TDM down-regulation in other pathogenic mycobacteria, borrowing host-derived glucose as an alternative substrate for their functional mycolyltransferases (*4*). Whereas the extrinsic pathway involves concomitant up-regulated expression of GMM, a specific target for host CD1b-restricted cytotoxic T cells that can detect and lyse mycobacteria-infected cells (*17*, *18*), the simultaneous down-regulation of TDM and GMM expression achieved by the intrinsic mechanism would minimize activation of both innate and acquired phases of host immunity, providing better chances for the microbe to survive in the host. Given that M.le is an obligate intracellular parasite that can survive only within the host cells, it might have been critically important for the bacteria to evolve highly efficient maneuvers for adaptation to host environments by even reducing the genuine function of mycolyltransferases. Transcription of *fbpA*, *fbpB* and *fbpC* genes could be detected during M.le growth (*19*). Nevertheless, its TDM expression is highly suppressed (*6*), implicating that the other mycolyltransferases, FbpB and FbpC, also fail to generate TDM. Unlike M.le FbpA that contains a unique amino acid at position 130, the corresponding amino acid residue in FbpB and FbpC is shared between M.le and M.tb. This suggests that distinct, but yet undetermined, mechanisms should have been evolved for M.le FbpB and FbpC that support TDM down-regulation.

As mentioned earlier, detailed lipid biochemical analysis of M.le has been hampered due to the limited sources of the bacteria. Nevertheless, the sequence of the recently decoded M.le genome (*7*) and its comparison with that of its close relative M.tb genome often provide a valuable clue that helps us to understand the yet undefined biology of M.le. The M.le genome is much smaller in size with more pseudogenes, resulting in

significantly reduced numbers of open reading frames that potentially encode functional proteins. It is noteworthy that, even after such a highly reductive evolution, the M.le genome still contains apparently functional *fbp* genes. Surprisingly, while specifically reducing the mycolyltransferase activity by blockade of the tunnel that accommodates the α-branch of mycolates, the M.le FbpA protein still preserves the molecular structure for interaction with the unbranched glycolipid substrates and the catalytic activity to transfer the unbranched acyl chain (Fig. 2). This suggests that the protein integrity of the M.le FbpA has been maintained during the reductive evolution and that it might play a role in the lifecycle of M.le, which is distinct from that as a mycolyltransferase. The present study, detecting differential mycolyltransferase activity in M.le and other pathogenic mycobacteria, provides a clue to the still mysterious biology of the first human pathogenic bacterium to be identified.

ACKNOWLEDGEMENTS

We thank Dr Shinji Maeda (Research Institute of Tuberculosis) for the gift of M.le-derived genomic DNA.

FUNDING

Grants from the Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid from Scientific Research on Priority Areas) (to M.S.); from the Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (B) (to M.S.) and (C) (to I.M.)]; from the Ministry of Health, Labour, and Welfare (Research on Emerging and Re-emerging Infectious Diseases) (to M.S.); the Shimizu Foundation Research Grant for 2007 (to I.M.).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Brennan, P.J. (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis* **83**, 91–97
2. Ryll, R., Kumazawa, Y., and Yano, I. (2001) Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids—a review. *Microbiol. Immunol.* **45**, 801–811
3. Otsuka, A., Matsunaga, I., Komori, T., Tomita, K., Toda, Y., Manabe, T., Miyachi, Y., and Sugita, M. (2008) Trehalose dimycolate elicits eosinophilic skin hypersensitivity in mycobacteria-infected guinea pigs. *J. Immunol.* **181**, 8528–8533
4. Matsunaga, I., Naka, T., Talekar, R.S., McConnell, M.J., Katoh, K., Nakao, H., Otsuka, A., Behar, S.M., Yano, I., Moody, D.B., and Sugita, M. (2008) Mycolyltransferase-mediated glycolipid exchange in Mycobacteria. *J. Biol. Chem.* **283**, 28835–28841
5. Belisle, J.T., Vissa, V.D., Sievert, T., Takayama, K., Brennan, P.J., and Besra, G.S. (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* **276**, 1420–1422
6. Dhariwal, K.R., Yang, Y.M., Fales, H.M., and Goren, M.B. (1987) Detection of trehalose monomycolate in *Mycobacterium leprae* grown in armadillo tissues. *J. Gen. Microbiol.* **133**, 201–209
7. Cole, S.T., Eiglmeier, K., Parkhill, J., James, K.D., Thomson, N.R., Wheeler, P.R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R.M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J.R., and Barrell, B.G. (2001) Massive gene decay in the leprosy bacillus. *Nature* **409**, 1007–1011
8. Maeda, S., Matsuoka, M., Nakata, N., Kai, M., Maeda, Y., Hashimoto, K., Kimura, H., Kobayashi, K., and Kashiwabara, Y. (2001) Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob. Agents Chemother.* **45**, 3635–3639
9. Raku, T., Kitagawa, M., Shimakawa, H., and Tokiwa, Y. (2003) Enzymatic synthesis of trehalose esters having lipophilicity. *J. Biotechnol.* **100**, 203–208
10. Kremer, L., Maughan, W.N., Wilson, R.A., Dover, L.G., and Besra, G.S. (2002) The *M. tuberculosis* antigen 85 complex and mycolyltransferase activity. *Lett. Appl. Microbiol.* **34**, 233–237
11. Matsunaga, I., Komori, T., Ochi, A., Mori, N., and Sugita, M. (2008) Identification of antibody responses to the serotype-nonspecific molecular species of glycopeptidolipids in *Mycobacterium avium* infection. *Biochem. Biophys. Res. Commun.* **377**, 165–169
12. Ogata, K. and Umeyama, H. (2000) An automatic homology modeling method consisting of database searches and simulated annealing. *J. Mol. Graph. Model* **18**, 258–272
13. Wheelock, C.E., Nakagawa, Y., Harada, T., Oikawa, N., Akamatsu, M., Smagghe, G., Stefanou, D., Iatrou, K., and Swevers, L. (2006) High-throughput screening of ecdysone agonists using a reporter gene assay followed by 3-D QSAR analysis of the molting hormonal activity. *Bioorg. Med. Chem.* **14**, 1143–1159
14. Anderson, D.H., Harth, G., Horwitz, M.A., and Eisenberg, D. (2001) An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (Antigen 85B), a mycolyl transferase. *J. Mol. Biol.* **307**, 671–681
15. Ronning, D.R., Klabunde, T., Besra, G.S., Vissa, V.D., Belisle, J.T., and Sacchettini, J.C. (2000) Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. *Nat. Struct. Biol.* **7**, 141–146
16. Ronning, D.R., Vissa, V., Besra, G.S., Belisle, J.T., and Sacchettini, J.C. (2004) *Mycobacterium tuberculosis* antigen 85A and 85C structures confirm binding orientation and conserved substrate specificity. *J. Biol. Chem.* **279**, 36771–36777
17. Moody, D.B., Reinhold, B.B., Guy, M.R., Beckman, E.M., Frederique, D.E., Furlong, S.T., Ye, S., Reinhold, V.N., Sieling, P.A., Modlin, R.L., Besra, G.S., and Porcelli, S.A. (1997) Structural requirements for glycolipid antigen recognition by CD1b- restricted T cells. *Science* **278**, 283–286
18. Stenger, S., Mazzaccaro, R.J., Uyemura, K., Cho, S., Barnes, P.F., Rosat, J.P., Sette, A., Brenner, M.B., Porcelli, S.A., Bloom, B.R., and Modlin, R.L. (1997) Differential effects of cytolytic T cell subsets on intracellular infection. *Science* **276**, 1684–1687
19. Williams, D.L., Torrero, M., Wheeler, P.R., Truman, R.W., Yoder, M., Morrison, N., Bishai, W.R., and Gillis, T.P. (2004) Biological implications of *Mycobacterium leprae* gene expression during infection. *J. Mol. Microbiol. Biotechnol.* **8**, 58–72

Lepr Rev (2009) **80,** 402–409

Detection of serum antibodies to *M. leprae* Major Membrane Protein-II in leprosy patients from Indonesia

MOCHAMMAD HATTA*, MASAHIKO MAKINO**, RATNAWATI*, MASHUDI*, YADI*, MUHAMMAD SABIR*, NATANIEL TANDIROGANG*, LUH MADE MAS RUSYATI***, MASANORI KAI**, YASUO FUKUTOMI**, YUJI MIYAMOTO**, TETSU MUKAI** & YUMI MAEDA**

**Department of Microbiology, Faculty of Medicine, Molecular Biology and Immunology Laboratory, Hasanuddin University, Km.10, Kampus Tamalanrea, Makassar, Indonesia*

***Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Higashimurayama, Tokyo, Japan*

****SMF Dermatology, Faculty Medicine, Sanglah Hospital, Denpasar, Bali, Indonesia*

Accepted for publication 23 September 2009

Summary

Background Sero-diagnostic methods are the easiest way of diagnosing an infectious disease in developing countries. In leprosy, phenolic glycolipid-1 (PGL-I) based methods for the detection of leprosy are currently available, but the use of these methods has been hindered due to the inherent problems of sensitivity. We previously showed that antibodies to Major Membrane Protein-II (MMP-II) derived from *Mycobacterium leprae* could be used to diagnose leprosy in Japan.

Methods Sera from patients and healthy individuals were collected with informed consent and the anti-MMP-II antibody levels of the sera were measured by enzyme-linked immunosorbent assay. The study was conducted at South Sulawesi and Bali, in Indonesia. The study population included 40 each of multibacillary leprosy and paucibacillary leprosy patients, 30 tuberculosis and 16 patients with typhoid.

Results We evaluated the anti-MMP-II antibody levels in Indonesian individuals. The cut-off value was determined from receiver operator characteristic curve as 0·124 using the O.D. titers for patients with multibacillary leprosy, so that the sensitivity of the test was 97·5% and the specificity taking healthy individuals as controls was 98·4%. Using the determined cut-off values, 98% of multibacillary (MB) leprosy and

Correspondence to: Yumi Maeda, Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan (Tel: +81-42-391-8211; Fax: +81-42-391-8212; e-mail: yumi@nih.go.jp)

0305-7518/09/064053+08 $1.00

48% of paucibacillary (PB) leprosy patients had positive levels of anti-MMP-II antibodies, 13% of patients with typhoid and 22% of the household contacts of MB leprosy had positive levels of anti- MMP-II antibodies.

Conclusions Our results suggest that measuring anti-MMP-II antibody levels could facilitate the detection of leprosy in endemic countries.

Introduction

Mycobacterium leprae, the causative agent of leprosy, induces skin lesions and peripheral nerve injuries which may lead to physical deformities.[1,2] Despite efforts by the World Health Organization to reduce the global leprosy burden, 254,525 new patients were detected during 2007, compared with 265,661 during 2006, which indicates that the progress towards complete elimination of leprosy is slow.[2] Timely detection of new patients and prompt treatment is the key to reducing the burden of leprosy. The most widely used antigen for serodiagnosis of leprosy is phenolic glycolipid-I (PGL-I).[3] Simple lateral flow test and dipstick assays, based on detecting the antibodies to the PGL-I antigen, have been developed to classify leprosy patients and identify contacts with an increased risk of developing leprosy.[4,5] A particle agglutination test has also been developed to detect anti-PGL-I antibodies qualitatively and quantitatively.[6,7] However, there are limitations in using these kits especially in terms of sensitivity. Numerous antigens of *M. leprae* have been tested for diagnostic feasibility,[8] but their use has been limited due to their poor performance. Duthie *et al.* performed a world-wide serological study using two *M. leprae* antigens namely ML0405 and ML2331, and found that these two proteins could be recognised by patients' sera from diverse regions.[9] We have previously identified Major Membrane Protein-II (MMP-II) from the cell membrane fraction of *M. leprae* as one of the antigenic molecules capable of activating both antigen-presenting cells and T cells.[10] When we evaluated the anti-MMP-II IgG levels in Japanese leprosy patients, 82·4% of MB leprosy and 39·0% of PB leprosy patients had positive results.[11] Here, we evaluated the use of MMP-II as a tool for sero-diagnosis of leprosy in Indonesia, which is an endemic countries with foci of high leprosy rates.

Material and Methods

Sera were obtained under informed consent from healthy individuals, leprosy patients, household contacts of leprosy patients, tuberculosis patients and typhoid patients from different parts of Indonesia. Sera were frozen at −30 °C until use. All patients who were clinically diagnosed were selected, so that there was no bias in the system, and double-blind test for ELISA was conducted. The population studied included multibacillary (MB) and paucibacillary (PB) leprosy patients, tuberculosis patients and typhoid patients. All patients diagnosed with tuberculosis had either acid fast bacilli in sputum smears stained with Ziel Neelsen stain and/or *M. tuberculosis* grown on culture. Patients diagnosed with typhoid had *S. typhimurium* grown on culture. All patients were newly diagnosed. We defined household contacts of leprosy patients ($n = 50$), as people living under the same roof and sharing food with the patient for at least 6 months. These contacts were collected either by home visits or in clinics from those family members who accompanied the patients.

Prior to the study, we received ethical clearance from the Ethical Research Committee of the Hasanuddin University and informed consent for participation in the study was obtained from all participants or their parents/guardians. We also received approval from the ethics committee of the National Institute of Infectious Diseases, Tokyo. Classification of leprosy was carried out according to Ridley-Jopling's classification and WHO recommendations (http://www.who.int/lep/classification/en/index.html).[12] Briefly, Ridley-Jopling's five group classification system is based on clinical, bacteriological and cytopathological findings, and WHO classification is based on clinical and skin smear test findings. Sera from *M. bovis* BCG-vaccinated healthy volunteers and blood donors ($n = 62$), who were local ethnic people from Makassar which is an endemic region in Indonesia, were collected from the Makassar blood transfusion unit and used as negative controls in ELISA tests to determine the cut-off.

The MMP-II protein used as antigen was purified as a fusion protein in *E. coli* using pMAL-c2X expression vector (New England BioLabs) as described earlier.[11] The ELISA for the detection of anti-MMP-II IgG antibodies was performed as described as follows: 96 well plates (Immunosorb, Nunc) were coated overnight with MMP-II at a concentration of 2 μg/ml in 0·1 M carbonate buffer (pH 9·5). After blocking with 2% skim milk in PBS (pH 7)(blocking solution), the plates were washed with phosphate buffered saline containing 0·1% Tween 20 (PBST) and human sera (normal, patients or household contacts) diluted 100 times in blocking solution, were added and incubated at 37 °C for 2 hours. After washing with PBST, biotinylated anti-human IgG (Vector Laboratories) in blocking solution was added at a dilution of 1:1500 and incubated for 1 hour. The plates were incubated with reagents from the ABC Kit (Vector Lab) in PBST for 30 min. After washing with PBST, substrate solution consisting of 0·2 mg/ml of OPD (o-phenylene diamine) and 0·02% H_2O_2 in 0·1 M citrate buffer (pH 5·5) was added until colour developed, after which the reaction was stopped with 0·5 M sulfuric acid. Optical density (O.D.) was measured using a Vmax spectrophotometer (Molecular Devices). Plate to plate variations in O.D. readings were controlled by using a common standard serum, and if this reading varied more than 0·05 O.D., then the test was repeated. For detecting anti-PGL-I antibodies, NTP-BSA was coated at a concentration of 0·5 μg/ml, and the same procedure used to detect anti-MMP-II antibodies was followed, except that the secondary antibodies used was biotinylated anti-human IgM (Vector Laboratories) at a dilution of 1:750.

For statistical analysis, Receiver Operator Characteristics (ROC) curves were drawn to describe the relation between sensitivity and specificity at various cut-off levels using the MedCalc software.

Results

The population studied included multibacillary (MB) ($n = 40$) and paucibacillary (PB) ($n = 40$) leprosy patients from several primary health care centers in South Sulawesi and Bali, tuberculosis patients ($n = 30$) from Tuberculosis Hospital, Makassar and typhoid patients ($n = 16$) from several primary health care centers in South Sulawesi. Sera from *M. bovis* BCG-vaccinated healthy volunteers and blood donors ($n = 62$) were used to determine the cut-off value for the assay. The anti-MMP-II IgG antibody levels (Mean O.D. ± S.D.) for MB leprosy, PB leprosy, tuberculosis, and healthy individuals were 0·488 ± 0·152, 0·139 ± 0·096, 0·038 ± 0·023 and 0·074 ± 0·023 respectively. The cut-off value of O.D. 0·124 was defined using a ROC curve, where the specificity of the test was 98·4%

Table 1. Evaluation of MMP-II-ELISA for serodiagnosis of leprosy in Indonesian individuals

Group	Total No.	No. of Positives	No. of Negatives	Percent Positivity	Mean O.D. values ± SD
MB leprosy	40	39	1	98	0·488 ± 0·152
PB leprosy	40	19	21	48	0·139 ± 0·096
Tuberculosis	30	0	30	0	0·038 ± 0·023
Typhoid	16	2	14	13	0·076 ± 0·132
Household Contacts	50	11	39	22	0·109 ± 0·079
Healthy	62	3	59	5	0·074 ± 0·023

Cut-off value of O.D. 0·124 was defined by using the ROC curve.

and the sensitivity was 97·5%. 39 out of 40 (98%) MB patients had positive antibody levels, and 19 out of 40 (48%) PB patients were positive for anti-MMP-II antibodies (Table 1).

None of the tuberculosis patients and two of the typhoid patients had antibodies against MMP-II. One of the typhoid patients had a very high titer of O.D. 0·543, which is comparable to those of patients with MB leprosy, and therefore this particular patient should be closely monitored for any clinical signs of leprosy. Only three of the 62 (5%) healthy individuals exhibited positive anti-MMP-II antibody levels. *P* values between the various groups in Table 1 were calculated by the comparison of means (student's t-test) as follows: *p* value was less than 0·0001 between MB and PB leprosy, PB and Healthy, MB and Healthy. When the anti-PGL-I IgM antibody levels were examined using the same leprosy patients, it was found that only 75% of MB patients and 28% of PB patients had positive values (Table 2).

The cut-off value of O.D. 0·214 was determined using a ROC curve, where the specificity of the test was 87·1% and the sensitivity was 75%. PGL-I IgM antibody levels in healthy individuals seem to be comparatively high, which increases the cut-off value. The antibody levels were significantly higher in MB patients than the healthy controls ($p < 0·0001$ for PGL-I). A comparison of antibody levels were in patients with PB leprosy and healthy controls showed that a MMP-II antibody levels were significantly higher $p < 0·0001$ than PGL-1 antibody levels ($p = 0·018$ (PGL-I)) These results may indicate that MMP-II based ELISA is superior to PGL-I ELISA.

In a serological survey of household contacts of 17 leprosy patients we found that 11 of 50 (22%) household contacts had positive anti-MMP-II antibody levels that were significantly

Table 2. Comparison of MMP-II and PGL-I based ELISA in Indonesian individuals

Group	Total No.	No. of Positives		Mean O.D. ± SD (PGL-I)
		MMP-II (IgG)	PGL-I (IgM)	
MB leprosy	40	39 (98%)	30 (75%)	0·345 ± 0·174
PB leprosy	40	19 (48%)	11 (28%)	0·158 ± 0·108
Healthy	62	3 (5%)	9 (15%)	0·114 ± 0·077

Cut-off value of O.D. 0·214 was defined by using the ROC curve.

Table 3. Anti-MMP-II-Ab O.D. of patients and their household contacts

Patient No.	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17
Patient Type	LL	LL	LL	BL	BL	BL	BL	BL	BL	BL	LL	BL	BL	BL	BL	BL	BL
BI value	4	4	4	3	3	3	3	3	3	2	2	2	2	2	1	1	1
O.D. value	**0·588**	**0·514**	**0·510**	**0·616**	**0·551**	**0·521**	**0·530**	**0·482**	**0·427**	**0·607**	**0·600**	**0·596**	**0·590**	**0·198**	**0·574**	**0·386**	0·106
Household contacts of each patient																	
No.1	0·078	0·103	**0·233**	0·102	0·121	**0·140**	0·117	**0·362**	0·096	**0·138**	0·036	**0·170**	**0·507**	**0·126**	0·045	0·123	**0·171**
No.2		0·099	**0·134**		0·063	0·115	0·084			0·082		0·094	**0·208**	0·080			0·115
No.3		0·062	**0·127**		0·055	0·082	0·069			0·036		0·077	0·081				0·088
No.4		0·061	0·118			0·078	0·044						0·049				
No.5		0·037	0·112			0·074											
No.6			0·106			0·062											
No.7			0·094			0·053											
No.8			0·092														
No.9			0·084														

O.D. values in bold represents those testing positive for anti-MMP-II antibodies.

higher ($p = 0{\cdot}0012$) than antibody levels in healthy controls. Table 3 shows the anti-MMP-II antibody titers of the patients arranged in descending order of the BI value and their household contacts.

Among the 17 leprosy patients included in our survey, eight had one or more household contacts with detectable anti-MMP-II antibodies. Patient #3, classified as LL (Bacterial Index = 4) had an O.D. titer of 0·510. Three of his nine household contacts, had detectable anti-MMP-II antibodies, although they had no clinical signs of leprosy.

Discussion

Elimination of leprosy necessitates not only treatment of leprosy by multi-drug therapy (MDT), but also early detection of patients. However, leprosy still remains a disease whose mode of transmission is unclear. Contact with MB patients is widely accepted to be responsible for the transmission of the bacilli, and the detection rate of new leprosy patients has not diminished. Thus, a survey for new cases especially in endemic regions is necessary to control the spread of the disease. For this purpose, the most easily performable method is the serological testing by a simple kit, but which antigen(s) are best suited for such a test is still not clear. A combinatorial approach using anti-phenolic glycolipid- I (PGL-I) and anti-45 kDa antibodies for detection of *M. leprae* infection seems to provide a method with higher specificity.[13] Also prevalence of antibodies to culture filtrate protein-10 is observed in 83·3% of BI-positive leprosy patients.[14] A survey of antibodies to these antigens in different leprosy endemic countries would provide further information for the diagnostic utility of these antigens in diagnosing leprosy. We previously identified MMP-II as one of the *M. leprae* components capable of stimulating $CD4^+$ and $CD8^+$T cells, and the protein was originally recognised to be identical to mycobacterial bacterioferritin.[10,15] Deshpande *et al.* reported that sera from leprosy patients have higher IgG titer to MMP-II, regardless of the clinical type of leprosy.[16] Recently, we performed a comparative study among Japanese leprosy patients and discovered that MMP-II is superior to PGL-I because it was able to detect 82% of multibacillary and 39% of paucibacillary leprosy.[11]

The present study included a population from a country geographically distinct from Japan, namely, Indonesia, in which leprosy is still endemic. There were 17 682 new patients detected in 2006, which places Indonesia among the top three countries having more than 10 000 new patients per year.[2] We evaluated the anti-MMP-II antibody titers in leprosy patients as well as non-leprosy patients, such as tuberculosis and typhoid patients, taking the healthy blood donors as controls, and found that 98% of MB patients and 48% of PB patients had detectable anti-MMP-II antibodies. The reason for the higher positive rate observed in Indonesian leprosy patients, may be partly due to the highly active disease state of the patients and because all patients were newly diagnosed, so that all sera were collected before the start of chemotherapy. When we evaluated the household contacts of patients, 22% of these household contacts were positive for the anti-MMP-II antibodies. One of the patients examined had no detectable anti-MMP-II antibodies, but one of his household contacts had a positive titer for anti-MMP-II antibodies. Again, these contacts should be carefully monitored serologically as well as for any other clinical signs of leprosy in order to promptly initiate chemotherapy. Also, attention should be given to patients who are HIV positive or immuno-compromised individuals, since their immune system may not produce sufficient antibodies for detection. In those grounds, ELISA tests are generally sensitive and specific, if a suitable

immunogenic antigen is available. But in leprosy endemic countries we need to develop more simple tests like the visibly detectable dip-stick test, but dip-stick tests are still not as sensitive, and therefore when the level of antibodies is low, the detection by a simple system is still not feasible.

This report indicates that MMP-II could contribute to the sero-detection of MB as well as PB leprosy patients in leprosy endemic countries, where other environmental mycobacterial infections may be more prevalent, such as in Indonesia. During the course of this study, we observed that in Vietnam 85% of MB patients and 48% of PB patients were positive for anti-MMP-II antibodies.[17] The development of sensitive, user-friendly tools for the detection of anti-MMP-II antibodies, may further contribute to the prompt detection of leprosy.

Acknowledgements

The synthetic bovine serum albumin (BSA)-conjugated trisaccharide-phenyl propionate (NTP-BSA) was kindly provided by Dr. T. Fujiwara from Nara University, for detection of PGL-I antibodies.

Financial support. The study was supported by the following grants: a Health Science Research Grant of Emerging and Re-emerging Infectious Diseases, the Ministry of Health, Labor and Welfare, Government of Japan; and partly by a grant from International Medical Center, the Ministry of Health, Labor and Welfare, Government of Japan.

References

[1] Job CK. Nerve damage in leprosy. *Int J Lepr Other Mycobact Dis*, 1989; **57**: 532–539.
[2] WHO. Global leprosy situation 2008. *Wkly Epidemiol Rec*, 2008; **33**: 293–300. http://www.who.int/wer.
[3] Fujiwara T, Aspinall GO, Hunter SW, Brennan PJ. Chemical synthesis of the trisaccharide unit of the species-specific phenolic glycolipid from *Mycobacterium leprae*. *Carbohydr Res*, 1987; **163**: 41–52.
[4] Buhrer-Sekula S, Cunha MG, Ferreira WA, Klatser PR. The use of whole blood in a dipstick assay for detection of antibodies to *Mycobacterium leprae*: a field evaluation. *FEMS Immunol Med Microbiol*, 1998; **21**: 197–201.
[5] Buhrer-Sekula S, Smits HL, Gussenhoven GC *et al.* Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. *J Clin Microbiol*, 2003; **41**: 1991–1995.
[6] Izumi S, Fujiwara T, Ikeda M *et al.* Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. *J Clin Microbiol*, 1990; **28**: 525–529.
[7] Hatta M, Izumi S, Klatser PR. Evaluation of the *Mycobacterium leprae* particle agglutination (MLPA) test as a tool in the epidemiology of leprosy in high prevalence village in South Sulawesi, Indonesia. *Southeast Asian J Trop Med Public Health*, 1995; **26**: 631–635.
[8] Hunter SW, Rivoire B, Mehra V *et al.* The major native proteins of the leprosy bacillus. *J Biol Chem*, 1990; **265**: 14065–14068.
[9] Duthie MS, Goto W, Ireton GC *et al.* Use of protein antigens for early serological diagnosis of leprosy. *Clin Vaccine Immunol*, 2007; **14**: 1400–1408.
[10] Maeda Y, Mukai T, Spencer J, Makino M. Identification of an immunomodulating agent from *Mycobacterium leprae*. *Infect Immun*, 2005; **73**: 2744–2750.
[11] Maeda Y, Mukai T, Kai M *et al.* Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy. *FEMS Microbiol Lett*, 2007; **272**: 202–205.
[12] Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis*, 1966; **34**: 255–273.
[13] Parkash O, Kumar A, Nigam A *et al.* Evaluation of recombinant serine-rich 45-kDa antigen (ML0411) for detection of antibodies in leprosy patients. *Scand J Immunol*, 2006; **64**: 450–455.

[14] Parkash O, Kumar A, Nigam A, Girdhar BK. Detection of antibodies against *Mycobacterium leprae* culture filtrate protein-10 in leprosy patients. *J Med Microbio*, 2006; **55**: 1337–1341.
[15] Pessolani MC, Smith DR, Rivoire B *et al.* Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. *J Exp Med*, 1994; **180**: 319–327.
[16] Deshpande RG, Khan MB, Bhat DA, Navalkar RG. Immunoaffinity chromatographic isolation of a high molecular weight seroreactive protein from *Mycobacterium leprae* cell sonicate. *FEMS Immunol Med Microbiol*, 1995; **11**: 163–169.
[17] Kai M, Nguyen Phuc NH, Hoang Thi TH *et al.* Serological diagnosis of leprosy in patients in Vietnam by enzyme-linked immunosorbent assay with *Mycobacterium leprae*-derived major membrane protein II. *Clin Vaccine Immunol*, 2008; **15**: 1755–1759.

Available online at www.sciencedirect.com

ScienceDirect

Procedia in Vaccinology 1 (2009) 127–134

Procedia in Vaccinology

www.elsevier.com/locate/procedia

2nd Vaccine Global Congress, Boston 2008

Induction of anti-tumor immune responses with oligomannose-coated liposomes targeting to peritoneal macrophages

Kunio Tsujimura[a,b,*], Yuzuru Ikehara[c,d], Toshi Nagata[e], Yukio Koide[f], Naoya Kojima[g]

[a]*Department of Infectious Diseases, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*

[b]*Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan*

[c]*Molecular Medicine Team of Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Central 2-12, Room 211, 1-1-1 Umezono, Tsukuba 305-8568, Japan*

[d]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan*

[e]*Department of Health Science, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*

[f]*Director, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*

[g]*Department of Applied Biochemistry, The Institute of Glycotechnology, Tokai University, 1117 Kitakaname, Hiratsuka 259-1292, Japan*

Abstract

We recently established a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) taken up by peritoneal phagocytic cells to carry anti-cancer drugs to milky spots known as a preferential metastatic site of gastric cancers (Ikehara *et al.* 2006. *Cancer Res.* 66: 8740-8748). In the present study, we applied this intraperitoneal DDS for systemic tumor immunotherapy employing ovalbumin (OVA) as a model antigen. The phagocytic cells ingesting the OMLs containing OVA (OML-OVA) injected into the peritoneal cavity were predominantly macrophages (Mϕ), as they showed adhesive characteristics and expressed F4/80 and CD11b almost exclusively. Peritoneal Mϕ taking up OML-OVA could activate OVA-specific $CD8^+$ (from OT-I: $OVA_{257-264}$/H-$2K^b$-specific) and $CD4^+$ (from OT-II: $OVA_{323-339}$/H-$2A^b$-specific) T cells much more effectively *in vitro* than those taking up soluble OVA. Furthermore, only the mice immunized with OML-OVA rejected E.G7-OVA (OVA-transfected EL4) but not EL4. These results indicate that the OMLs can also be used as an effective antigen delivery system for tumor immunotherapy activating both $CD8^+$ and $CD4^+$ T cell subsets.

Key words: Oligomannose-coated liposome; Antigen delivery system; Tumor immunotherapy; Macrophage

* Corresponding author. Kunio Tsujimura Tel.: +81-53-435-2335; fax: +81-53-435-2335.
E-mail address: ktsujimu@hama-med.ac.jp

1877-282X/$–see front matter © 2009 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.provac.2009.07.024

1. Introduction

Recent advances in tumor immunology enable us to identify tumor antigens recognized by T cells and understand the molecular and cellular bases of T cell-mediated anti-tumor responses (1, 2). However, there are several problems left for the establishment of effective immunotherapy against solid tumors. Many $CD8^+$ and $CD4^+$ T cells recognizing tumor antigens in the context of MHC class I and II, respectively, have been reported, and the former are known to be a major effector of the adaptive anti-tumor immune responses (3-5). $CD4^+$ T cells play an important role for the expansion and persistence of $CD8^+$ T cells, while some of them are known to function as regulatory cells (5-7). Optimal anti-tumor immune responses are therefore considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the selective activation of $CD4^+$ T cells with helper but not regulatory functions (8). In general, endogenous and exogenous antigens are presented as peptides preferentially by MHC class I and II, respectively, and most tumor antigen peptides are derived from the proteins expressed endogenously. Novel methods to induce effective antigen presentation by MHC class I and II molecules simultaneously are therefore needed for the concomitant activation of tumor antigen-specific $CD8^+$ and $CD4^+$ T cells, and many attempts have been made for this purpose (2, 3, 8).

We recently developed a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) (9, 10) which are effectively taken up by $F4/80^+$ peritoneal cells to carry anti-cancer drugs to milky spots known as a preferential metastatic site of gastric and ovarian cancers (9). We demonstrated that this system could control the formation of overt metastasis of seeded gastric cancer cells at the extra-nodal lymphoid tissues such as the omentum (10-12).

In the present study, we applied this OML-based intraperitoneal DDS for tumor immunotherapy using ovalbumin (OVA) as a model antigen, aiming at the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells. Peritoneal macrophages (Mϕ) took up OMLs containing OVA (OML-OVA) and activated both OVA-specific $CD8^+$ and $CD4^+$ T cells effectively *in vitro*. In addition, OML-OVA immunized mice rejected tumor cells expressing OVA but not their parental cells. These results together indicate the potential of our novel OML-based immunization for the development of effective tumor immnotherapy.

2. Materials and methods

Mice. Female C57BL/6 (B6) mice at 8-12 weeks of age were obtained Charles River Japan Inc. (Yokohama, Japan). T cell receptor (TCR) transgenic mice OT-I (specific for $OVA_{257-264}$ peptide presented by $H\text{-}2K^b$) (13, 14) and OT-II (specific for $OVA_{323-339}$ peptide presented by $H\text{-}2A^b$) (15) were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Aichi Cancer Center.

Cell lines. EL4 (16), a B6-derived thymoma cell line, was maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum, 0.2% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (complete RPMI). E.G7-OVA (EL4 transfected with *OVA* gene) (17) was obtained from ATCC (Manassas, VA) and maintained in complete RPMI supplemented with 400 μg/ml G418 (Wako, Osaka, Japan) in a humidified 5% CO_2 incubator at 37°C.

Man3-DPPE and liposome preparation. Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma-Aldrich (St. Louis, MO). Mannotriose (Man3: Manα1-6(Manα1-3)Man) was purchased from Funakoshi (Tokyo, Japan). Man3-DPPE was prepared by conjugation of the mannotriose with DPPE by reductive amination as described previously (10, 18). The purity of Man3-DPPE was confirmed by high-performance thin-layer chromatography (Silica gel 60 HPTLC plate, MERCK, Darmstadt, Germany) and time-of-flight mass spectrometry (Auto FLEX, Bruker Daltonics, Bremen, Germany). The purified Man3-DPPE was quantified by determination of the phosphate contained.

OMLs were prepared as described previously (10). Briefly, a chloroform-methanol (2:1, v/v) solution containing 1.5 μmol of DPPC and 1.5 μmol of cholesterol was placed in a conical flask and dried by rotary evaporation. Subsequently, 2 ml ethanol containing 0.15 μmol of Man3-DPPE was added to the flask and evaporated to prepare a lipid film containing neoglycolipids. Procedures for protein-encasing into OMLs were performed as described

previously (10). The multilamellar vesicles were generated with either 200 μl of FITC-labeled or non-labeled OVA (5.0 mg/ml, Sigma-Aldrich) in the dried lipid film by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through polycarbonate membranes of 1 μm pore (Nucleopore, Pleasanton, CA). OMLs entrapping proteins were separated from free proteins by four successive rounds of washing in PBS with centrifugation (20,000 x *g*, 30 min) at 4°C. The amounts of entrapped proteins were measured using a modified Lowry protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL) in the presence of 0.3% (w/v) sodium dodecyl sulfate using bovine serum albumin as a standard.

Flow cytometry. One hour after intraperitoneal injection of OMLs containing FITC-OVA, peritoneal cells were recovered from B6 mice with 5 ml ice,cold PBS. Peritoneal cells were incubated on ice for 30 min with PE-labeled antibodies after blocking with mouse Fc Blocker (BD Biosciences, San Jose, CA) and then analysed on a FACSCalibur (BD Biosciences). The following monoclonal antibodies used in this study were purchased or kindly provided: anti-F4/80 (A3-1, Serotec Ltd., Oxford, UK), anti-MHC class II (M5/114.15.2, e-Bioscience, Boston, MA), anti-CD11b (M1/70.15, Caltag Laboratories, Burlingame, CA), anti-CD3ε (145-2C11, BD Biosciences), anti-CD19 (1D3, BD Biosciences), and anti-H-2K^bD^b (20-8-4S, Dr. E. Nakayama, Okayama University).

***In vitro* activation of OVA-specific T cells.** One hour after injection of either OML-OVA or soluble OVA into the peritoneal cavity of B6 mice, peritoneal cells were recovered with 5 ml ice cold PBS. The suspended peritoneal cells in complete RPMI were seeded into a 96-well culture plate (5 x 10^5 cells in each well) and incubated overnight at 37°C in a humidified 5% CO_2 incubator. On the next day, non-adherent cells were washed out with complete RPMI, and the adherent cells preferentially consisting of Mϕ were co-cultured with 5 x 10^5 cells of either $CD8^+$ or $CD4^+$ T cells from the spleen of OT-I and OT-II mice, respectively. $CD8^+$ and $CD4^+$ T cells were prepared with the isolation kits for corresponding subsets (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The supernatants were collected at 24 h and assayed for IFN-γ production with Mouse IFN-γ ELISA kit (Pierce).

***In vivo* tumor growth.** B6 mice were immunized three times biweekly by peritoneal injection of 1 μg OML-OVA. One week after the final immunization, tumor cells were injected subcutaneously into the backs of mice, and the survival time was observed.

3. Results

OMLs are taken up preferentially by peritoneal Mϕ. We showed that OMLs are incorporated very effectively by peritoneal cells expressing F4/80 and that the OML-ingesting cells are very useful drug delivery vehicles for tumor chemotherapy (9, 10). To verify whether the OMLs are applicable also for tumor immunotherapy, we first analyzed the $F4/80^+$ peritoneal cells in detail. FITC-labeled OVA was encased in OML and injected into the peritoneal cavity of B6 mice. One hour after the OML injection, peritoneal cells were collected and analyzed. As shown in Figure 1, peritoneal cells were divided into three groups based on the incorporation of OMLs. Most OVA^{high} cells expressed F4/80 and CD11b but not CD3 and CD19, and this population could be removed by plastic adherence. These observations together suggest that OVA^{high} population preferentially consists of Mϕ. The peritoneal cells with lower OML uptake (OVA^{low}) did not express F4/80, and nearly 2/3 of them were considered to be B cells because of their CD19 expression.

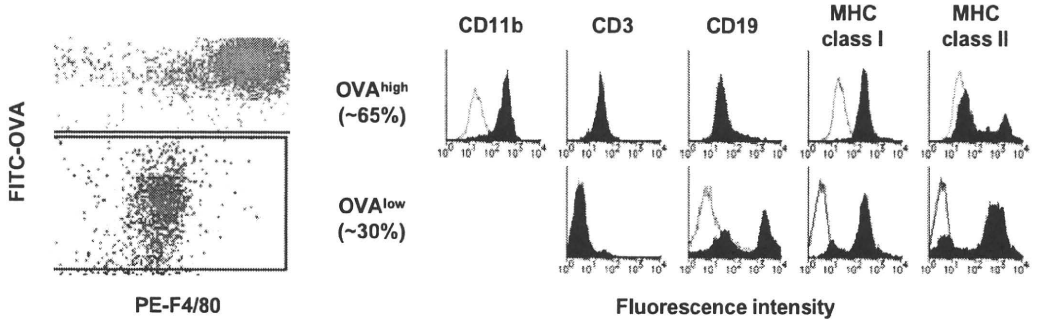

Figure 1. Surface phenotype of peritoneal cells from OML-injected mice. One hour after the injection of OMLs containing FITC-labeled OVA, peritoneal cells were collected and their surface phenotype was analyzed by flow cytometry.

Peritoneal Mϕ ingesting OML-OVA activate both CD8$^+$ and CD4$^+$ T cells in vitro in an antigen-specific manner. We next analyzed the antigen-presenting capacity of the peritoneal Mϕ ingesting OML-OVA. CD8$^+$ T cells from OT-I (OVA$_{257-264}$/H-2K^b-specific) and CD4$^+$ T cells from OT-II (OVA$_{323-339}$/H-2A^b-specific) were used as responder cells. When these T cells were co-cultured with peritoneal Mϕ from the mice intraperitoneally injected with OML-OVA, both CD8$^+$ and CD4$^+$ T cells produced large amounts of IFN-γ (Figure 2). Although Mϕ from the mice injected with soluble OVA also stimulated both CD8$^+$ and CD4$^+$ T cells, much higher amounts of OVA were needed compared to those from the mice injected with OML-OVA. We also found that cytotoxic T lymphocytes (CTL) against E.G7-OVA can be effectively induced in B6 mice immunized with OML-OVA (data not shown).

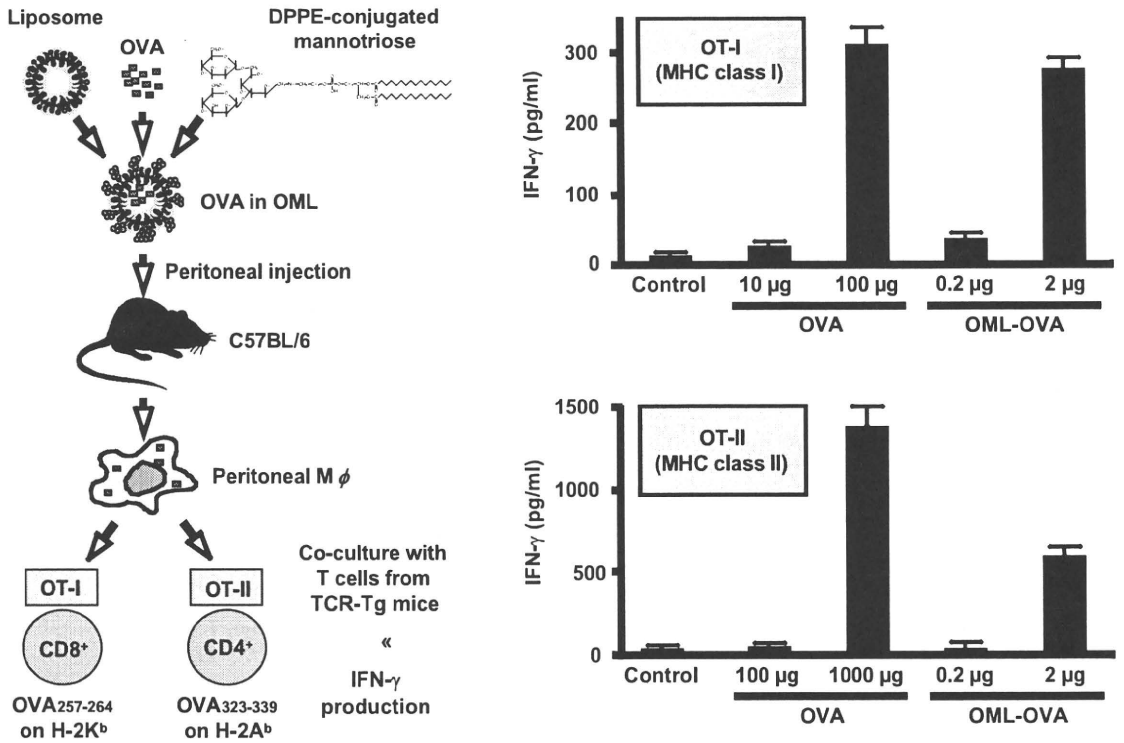

Figure 2. Peritoneal Mϕ ingesting OML-OVA activate OVA-specific CD8$^+$ and CD4$^+$ T cells much more effectively than those ingesting soluble OVA. One hour after intraperitoneal injection of antigens, peritoneal cells were prepared from the immunized mice, and Mϕ were enriched by plastic adherence. CD8$^+$ and CD4$^+$ T cells were purified from the spleen of OT-I and OT-II, respectively, and co-cultured with peritoneal Mϕ for 24 h. The supernatants were then collected and assayed for IFN-γ production by ELISA. Peritoneal Mϕ obtained from naïve mice were also used as a control.

OML-mediated immunization induces antigen-specific anti-tumor immunity in vivo. We finally examined whether intraperitoneal immunization with OMLs also induces antigen-specific anti-tumor immunity *in vivo*. Mice were immunized intraperitoneally with OML-OVA and then challenged subcutaneously with E.G7-OVA or EL4. As shown in Figure 3, only the immunized mice survived for more than 70 days when challenged with E.G7-OVA, while naïve mice died within 55 days. All the mice including those immunized with OML-OVA died within 30 days when challenged with EL4, indicating that the rejection of E.G7-OVA is OVA-specific. These results

together show that OML-mediated immunization can induce systemic immune response robust enough to protect mice from tumor challenge in an antigen-specific manner.

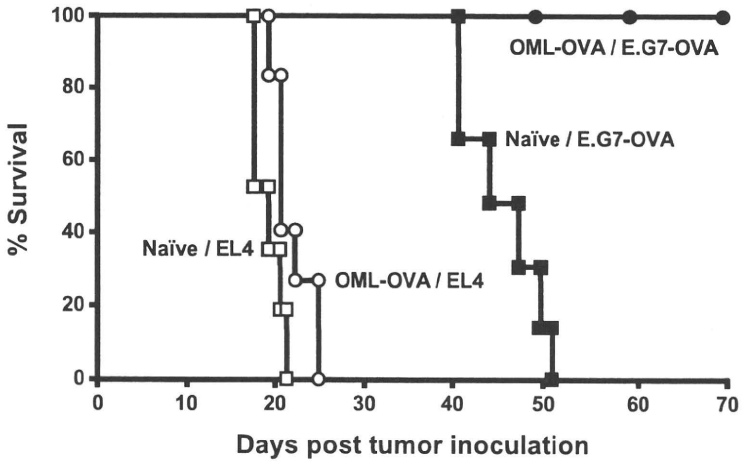

Figure 3. OML-mediated immunization induces antigen-specific anti-tumor immunity *in vivo*. B6 mice were immunized three times biweekly by peritoneal injection of 1 μg OML-OVA. One week after the last challenge, OML-OVA immunized (circles) and naïve (squares) mice were challenged subcutaneously with E.G7-OVA (closed symbols) or EL4 (open symbols).

4. Discussion

In this study, we demonstrated that our novel OML-based DDS targeted to peritoneal Mϕ can also be used for the induction of systemic immune responses. After ingesting OML-OVA, Mϕ migrate to extra-nodal lymphoid tissues in abdominal cavity and present OVA-derived peptides in the context of both MHC class I and II molecules. Only the mice immunized with OML-OVA rejected the challenge of E.G7-OVA but not EL4. These results together indicate that the OMLs can be used as an effective antigen delivery system for tumor immunotherapy activating both $CD8^+$ and $CD4^+$ T cell subsets as well as a DDS for tumor chemotherapy (Figure 4).