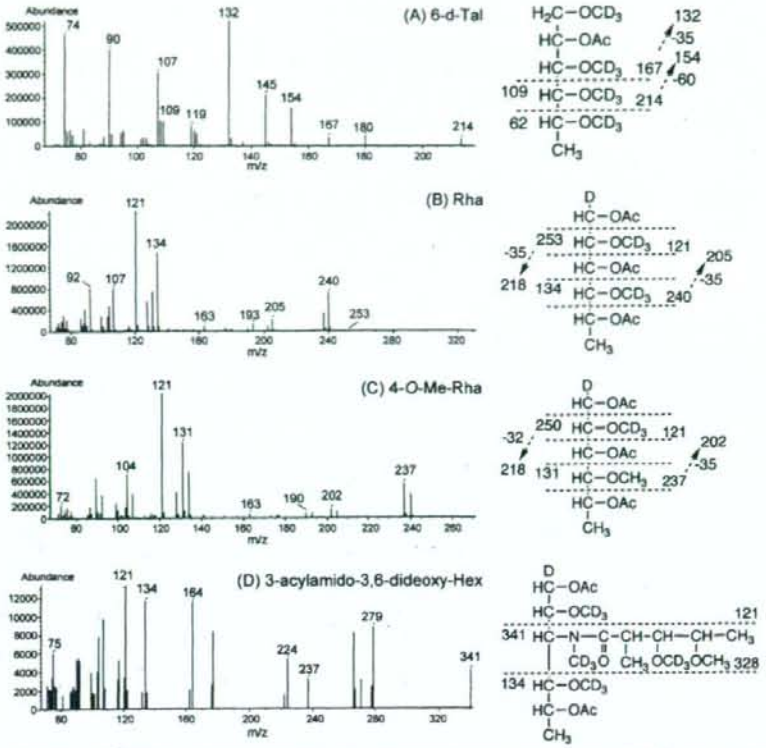

FIG. 4. GC-MS spectra of individual perdeuteromethylated alditol acetate derivatives from serotype 16 OSE. The formation of prominent fragment ions is illustrated; fragments were assigned to 1,3,4,5-tetra-*O*-deuteromethyl-2-*O*-acetyl-6-deoxy-talitol (A), 2,4-di-*O*-deuteromethyl-1,3,5-tri-*O*-acetyl-rhamnitol (B), 2-*O*-deuteromethyl-4-*O*-methyl-1,3,5-tri-*O*-acetyl-rhamnitol (C), 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 (D).

type 16 ATCC 13950^T was deposited in the NCBI GenBank database (accession no. AB355138). The similarity to protein sequences of each ORF is summarized in Table 1, and the genetic map for the serotype 16 GPL biosynthetic cluster was compared with those of serotype 2, 4, and 7 GPLs (Fig. 7). The *gtfB* and *drrC* genes of *M. intracellulare* serotype 16 ATCC 13950^T had 99.8% and 83.7% DNA identities with those of *M. intracellulare* serotype 7 ATCC 35847, respectively. In the DNA region between *gtfB* and *drrC* (20.8 kb), 17 ORFs were observed. Four ORFs (ORF 1, 2, 16, and 17) were homologous to those found in the same region of serotype 7-specific DNA, and the others were unique to the serotype 16 strain. No insertion of insertion elements or transposons was detected in this region. The nucleotide sequences of the ORF 1 and ORF 2 in serotype 16 strain ATCC 13950^T were homologous to those of ORF 1 and ORF 8 in serotype 7, respectively, suggesting that these two ORFs have the same function. The similarity of the deduced amino acid sequences suggested the possibility that the functions of ORF 3 and ORF 6 are to encode methyltransferase and aminotransferase, respectively. The deduced amino acid sequences of ORF 4 and ORF 5 showed significant similarities to the WxcM protein, the function of which is not clear. Interestingly, the deduced amino acid sequences of ORF 16 and ORF 17 of serotype 16 were homologous to ORF 9 of serotype 7. ORFs 1, 16, and 17 have considerable homology to glycosyltransferases. Nine ORFs, which are possibly involved in fatty acid synthesis, were detected between ORF 7 and ORF 15. It is notable that ORF 13 had a chimeric structure. The N-terminal half of ORF 13 showed similarity to phosphate butyryl/acetyl transferases, but the C-terminal half showed similarity to short-chain reductase/dehydrogenases. These results suggest that this region of DNA is responsible for the biosynthesis of the serotype 16-specific GPL.

Expression of cosmid clone no. 253 in *M. avium* serotype 1 strain. The OSE of serotype 1 GPL was composed of α-L-Rha-

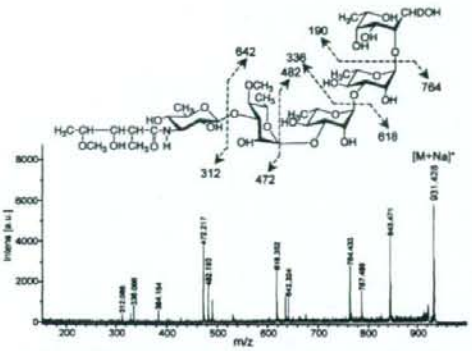

FIG. 5. MALDI-TOF/TOF MS spectrum of serotype 16 OSE. The formation of a characteristic increment in fragment ions is illustrated. The matrix was 10 mg/ml 2,5-dihydroxybenzoic acid in ethanol-water (3:7, vol/vol), and it was performed in the lift-lift mode. Intens., intensity; a.u., absorbance units.

(1→2)-6-d-L-Tal (9). The *M. avium* serotype 1 strain (NF113) was transformed with cosmid clone no. 253 containing a serotype 16-specific gene cluster and produced a new GPL with a different R_f value by TLC compared to serotype 1 GPL (Fig. 8A). The R_f value of the new GPL was identical to that of the serotype 16 GPL. The molecular weight of intact GPL, the fragment pattern of its OSE, and the GC pattern of the alditol acetate derivatives were completely equivalent to those of the serotype 16 GPL (see Fig. S2 in the supplemental material). As a result, the transformant of the serotype 1 strain expressed the cosmid clone no. 253 gene cluster and produced the serotype 16 GPL.

DISCUSSION

MAC species have serotype-specific GPLs that are characteristic components of the outer layer of the cell wall (6, 9). In addition to their serological differentiation, the chemical structures of 15 serotype-specific GPLs derived from the predominant clinical isolates have been analyzed; however, those of other GPLs remain unclear. The present study demonstrates the chemical structure of the serotype 16 GPL derived from *M. intracellulare*. We determined the glycosyl composition, linkage positions, and anomeric and ring configurations of the glycosyl residues of the serotype 16 GPL, and its OSE was defined as 3-2′-methyl-3′-hydroxy-4′-methoxy-pentanoyl-amido-3,6-dideoxy-β-Hex-(1→3)-4-*O*-methyl-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-6-d-α-L-Tal (Fig. 8B). The serotype 16 GPL should be listed as a group 2 polar GPL in the structural classification of Chatterjee and Khoo (9).

The GPLs of serotypes 7, 12, 17, and 19 have already been classified as group 2 GPLs, which are commonly composed of R→α-L-Rha-(1→3)-α-L-Rha-(1→2)-6-d-L-Tal (R, variable region), possessing a characteristic terminal sugar such as *N*-acyl-deoxy-Hex. Indeed, the presence of an amido sugar has been reported in only five GPLs, serotypes 7, 12, 14, 17, and 25 (8, 9, 18). It has been determined that the OSE structure of the

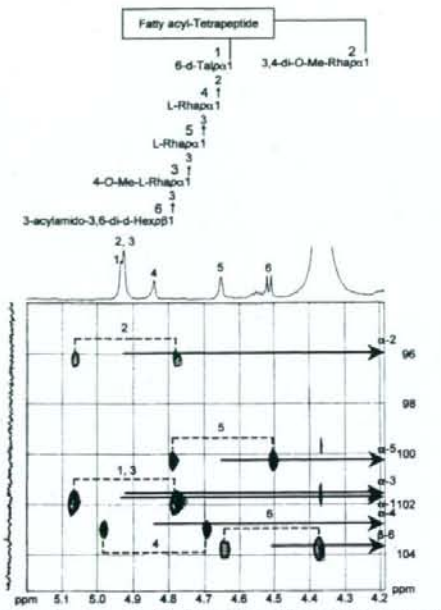

FIG. 6. Nondecoupled ^{1}H-detected [^{1}H, ^{13}C] HMQC spectrum of serotype 16 GPL. Cross-peak labels correspond to those shown on the structure.

serotype 17 GPL was 3-2′-methyl-3′-hydroxy-butanoyl-amido-3,6-dideoxy-β-D-Glc-(1→3)-4-*O*-methyl-α-L-Rha-(1→3)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-6-d-L-Tal (9, 25). Based on the behavior of GPLs in TLC and the GC-MS analysis of alditol acetate derivatives, serotype 16 GPL seems to possess a unique carbohydrate epitope similar to that of serotype 17 GPL. We compared the OSE of serotype 16 GPL to that of serotype 17 GPL. The acylated amido group that was bound to the terminal sugar was different, although the linkage position was identical. Except for the terminal-acylated amido sugar, the other sugar compositions and glycosyl linkage positions were completely identical. An acylated amido group attached to the C-3 position of Hex is very unusual. To our knowledge, 3-amido-Hex is irregular in nature, although 2-amido-Hex is known to be glucosamine or galactosamine, which is frequently isolated as a component of lipopolysaccharides and glycosaminoglycans in prokaryotic and eukaryotic cells (7, 42). Further, existence of short-chain fatty acid 2-methyl-3-hydroxy-4-methoxy-pentanoic acid linked to the amido group of d-Hex is also unique. The characteristic gene cluster is thought to regulate the production of 3-acylated-amido-Hex. It is difficult to determine the species of acylated amido sugars, because no reference standard is available. The terminal sugar of the serotype 17 GPL was reviewed as a gluco-configuration, although firm evidence was not shown (9, 25). The J_{CH} and $J_{1,2}$ values for the anomeric proton in the terminal sugar were 161 and 7.7 Hz,

TABLE 1. Similarity to protein sequences of ORFs in cosmid clone no. 253 derived from *M. intracellulare* serotype 16 strain ATCC 13950^T

ORF	Predicted molecular mass (kDa)	Predicted pI	Exhibits similarity to:	E value	Amino acid identity (no. matched/total no.)	Accession no.
GtfB	45.6	6.35	Glycosyltransferase GtfB	0.0	417/418	BAF45360
Orf 1	45.2	6.10	Putative glycosyltransferase	0.0	416/417	BAF45361
Orf 2	78.9	8.51	Putative acyltransferase	0.0	557/728	BAF45368
Orf 3	31.0	5.88	Putative methyltransferase	2e-89	382/421	NP_218045
Orf 4	15.7	4.94	Conserved hypothetical protein	1e-39	73/129	BAD50406
Orf 5	16.0	4.69	Conserved hypothetical protein	5e-40	75/135	EAX55190
Orf 6	41.1	5.88	Aminotransferase/DegT_DnrJ_EryC1	6e-119	208/357	ABD68440
Orf 7	40.6	9.65	Conserved hypothetical protein	2e-89	178/304	AAS03547
Orf 8	36.7	5.32	Conserved hypothetical protein	2e-52	116/298	CAE06954
Orf 9	22.3	9.79	Putative *N*-acetyltransferase	4e-14	58/166	EAU11841
Orf 10	25.3	7.82	Short-chain dehydrogenase/reductase	7e-47	101/233	EAO61220
Orf 11	23.8	6.05	Putative hydrolase	4e-24	64/196	ABG85599
Orf 12	37.2	6.50	Ketoacyl-acyl carrier protein synthase III	3e-55	126/331	EAX48715
Orf 13	42.5	7.72	Short-chain dehydrogenase/reductase	2e-42	97/248	ZP_01289005
Orf 14	65.8	4.70	Predicted enzyme involved in methoxymalonyl-acyl carrier protein biosynthesis	6e-85	201/575	ABB73590
Orf 15	50.0	6.23	Acyl coenzyme A synthetases	2e-128	233/445	EAT27362
Orf 16	39.1	8.00	Putative glycosyltransferase	2e-106	196/318	NP_855197
Orf 17	37.7	9.46	Putative glycosyltransferase	8e-160	278/323	BAF45369
DrrC	28.6	11.47	Daunorubicin resistance protein C	6e-132	233/261	BAF45370

respectively (Fig. 6; Table S1 in the supplemental material). These results demonstrated unequivocally that the terminal amido-Hex was β configuration and H-2 was in the axial position. The terminal amido-Hex is considered to be derived from glucose or galactose, not Rha.

Next, we explored the genetic mechanism of GPL biosynthesis, because the elongation of carbohydrate chains in serotype-specific GPLs is poorly understood. The *ser2* gene cluster of the *M. avium* serotype 2 strain (31) and a 27.5-kb DNA fragment of the *M. avium* serotype 4 strain (28) were identified to be responsible for the biosynthesis of each OSE in GPLs. Recently, enzymatic characterizations of glycosyltransferase and methyltransferase of nonpolar GPLs have been reported for *Mycobacterium smegmatis* (36, 38). In the serotype-specific polar GPL biosynthesis of MAC, only the *rtfA* gene was functionally clarified to encode the transfer of L-Rha to 6-d-Tal, but which gene cluster transfers the sugars next to L-Rha elongated from 6-d-Tal is unclear.

In this study, we cloned the biosynthetic cluster of the serotype 16 GPL and analyzed its sequence. Seventeen ORFs were detected in the serotype 16 strain, and the sequence homology was analyzed. The transformant of the *M. avium* serotype 1

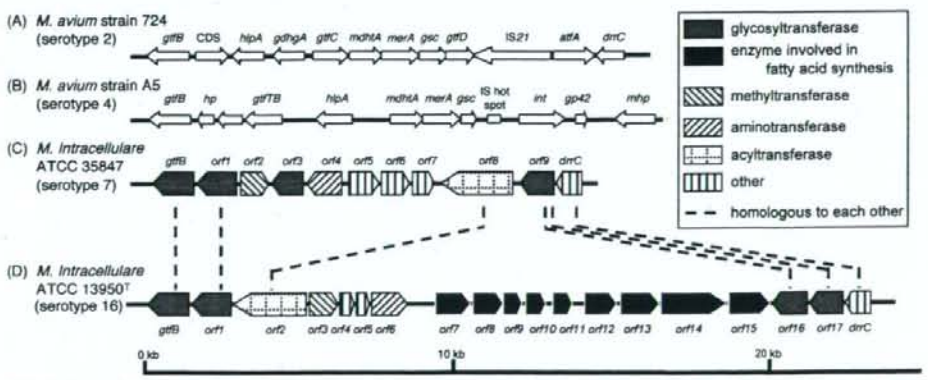

FIG. 7. Comparison and overview of genetic maps of GPL biosynthetic cluster. The *M. avium* strain 724 annotated sequence obtained from GenBank (accession no. AF125999) (A); the *M. avium* strain A5 annotated sequence obtained from GenBank (accession no. AY130970) (B); the *M. intracellulare* ATCC 35847 sequenced in our previous study (GenBank accession no. AB274811) (C); the *M. intracellulare* ATCC 13950^T sequenced in this study (GenBank accession no. AB355138) (D). The orientation of each gene is shown by the direction of the arrow. In panels A and B, putative ORFs not showing homology to known proteins sequences are not depicted. The sequences extending upstream in panels A and B and downstream in panel B are not included in the figure.

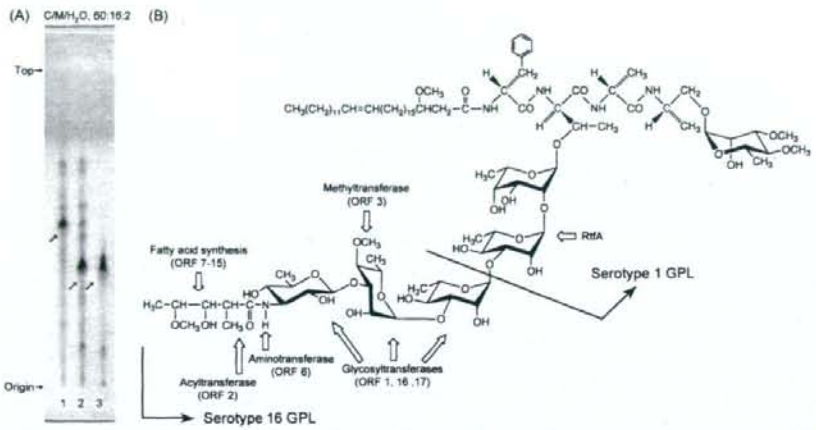

FIG. 8. TLC pattern of *M. avium* serotype 1 and its transformant with cosmid clone no. 253 and proposed complete structure of the serotype 16 GPL. (A) The alkaline-stable lipids derived from *M. avium* serotype 1 (lane 1), its transformant (lane 2), and purified serotype 16 GPL (lane 3) were developed with the solvent system of chloroform-methanol-water (60:16:2, vol/vol/vol). (B) Predicted biosynthesis gene clusters are indicated by arrows.

strain carrying cosmid clone no. 253 produced serotype 16 GPL. These results strongly implied that this *gtfB-drrC* region is responsible for the biosynthesis of the serotype 16-specific GPL. From the structural analysis of the serotype 16 GPL and the sequence of cosmid clone no. 253, it is possible to predict the relationship between the biosynthesis of serotype 16 GPL and the function of each ORF.

The genetic map of the serotype 16 GPL biosynthetic cluster was compared to those of serotype 2 GPL from *M. avium* strain 724, serotype 4 GPL from *M. avium* strain A5, and serotype 7 GPL from *M. intracellulare* strain ATCC 35847^T (12, 18, 28). Significant differences were found in the neighborhood of the conserved region. The genetic organization of the serotype 16 GPL gene cluster was distinct from that of serotype 7, except for some of the ORFs, and the ORFs in this region of serotype 2 and serotype 4 were completely different from ORFs 1 to 17 in serotype 16 (Fig. 7).

In addition to *M. intracellulare* serotype 7 (18) and serotype 16 strains, we have analyzed similar gene clusters of *M. intracellulare* serotype 12 and 17 strains. The sequence homology of the regions of ORF 1 and ORF 17 was highly conserved between only *M. intracellulare* serotype 16 and 17 strains (unpublished data). ORFs 1, 16, and 17 may lead to transfer of the two additional molecules of L-Rha and terminal amido-Hex. ORF 2 was assigned to acyltransferase and may be responsible for biosynthesis of the 3-2'-methyl-3'-hydroxy-4'-methoxy-pentanoyl-amido group in the terminal Hex. ORF 3 is probably responsible for the transfer of the *O*-methyl group at the C-4 position in the third L-Rha from 6-d-Tal. ORF 6 is homologous to aminotransferase and possibly associated with the biosynthesis of an amido group in the terminal Hex. The deduced amino acid sequences of ORF 6 in serotype 16 and ORF 4 in serotype 7 have homologies to DegT_DnrJ_EryC1 aminotransferases. However, these two ORFs are dissimilar to each other. Serotype 16 and 7 GPLs have an amido group at the terminal Hex, although the attachment position is different. The serotype 7 GPL has an amido group at the C-4 position in the terminal Hex, but the serotype 16 GPL has it at the C-3 position. Nine ORFs between ORF 7 and ORF 15 are possibly involved in fatty acid synthesis of the acyl chain moiety linked by an amido bond of the terminal Hex. Taken together, this gene cluster may participate in the biosynthetic pathway of the serotype 16-specific GPL, although further study is needed to clarify the function of each ORF.

Recent studies suggest that GPLs play an important role in the phenotype and pathogenicity of MAC. The colony morphology is considered to be influenced by cell wall GPL. MAC colony phenotypes spontaneously occur from smooth to rough type, and this is due to a mutation lacking GPL (3, 13, 22). The deletion of genomic regions encoding GPL biosynthesis may result in the loss of GPL. Danelishvili et al. demonstrated that the uptake by and growth in macrophages of a MAC mutant with the gene belonging to the GPL synthesis pathway inactivated by transposon insertion were decreased (11). Bhatnagar and Schorey have reported that macrophages infected with MAC release exosomes containing GPLs that result in the transfer of the GPLs to uninfected macrophages and induce a proinflammatory response (4). These findings imply that GPL participates in the pathogenicity of MAC. By contrast, our previous studies have demonstrated that anti-GPL antibodies are detected in the sera of most immunocompetent patients with MAC pulmonary disease and that the detection of anti-GPL antibody is useful for the serodiagnosis of MAC disease (15, 26, 27).

To understand the role of GPLs in MAC and its hosts, it is necessary to define the chemical structure and biosynthesis pathways of GPLs. Elucidation of the structure-function relationship of GPL may open a new avenue for controlling MAC disease.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Japan Health Sciences Foundation, and the Ministry of Health, Labor, and Welfare of Japan (Research on Emerging and Reemerging Infectious Diseases).

We are grateful to Sumihiro Hase (Department of Chemistry, Graduate School of Science, Osaka University, Osaka, Japan) and Hiromi Murakami (Osaka Municipal Technical Research Institute, Osaka, Japan) for helpful discussion.

REFERENCES

1. **Baess, I.** 1983. Deoxyribonucleic acid relationships between different serovars of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. Acta Pathol. Microbiol. Immunol. Scand. **91**:201–203.
2. **Barrow, W. W., T. L. Davis, E. L. Wright, V. Labrousse, M. Bachelet, and N. Rastogi.** 1995. Immunomodulatory spectrum of lipids associated with *Mycobacterium avium* serovar 8. Infect. Immun. **63**:126–133.
3. **Belisle, J. T., K. Klaczkiewicz, P. J. Brennan, W. R. Jacobs, Jr., and J. M. Inamine.** 1993. Rough morphological variants of *Mycobacterium avium*. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. J. Biol. Chem. **268**:10517–10523.
4. **Bhatnagar, S., and J. S. Schorey.** 2007. Exosomes released from infected macrophages contain *Mycobacterium avium* glycopeptidolipids and are proinflammatory. J. Biol. Chem. **282**:25779–25789.
5. **Bhatt, A., N. Fujiwara, K. Bhatt, S. S. Gurcha, L. Kremer, B. Chen, J. Chan, S. A. Porcelli, K. Kobayashi, G. S. Besra, and W. R. Jacobs, Jr.** 2007. Deletion of *kasB* in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. Proc. Natl. Acad. Sci. USA **104**:5157–5162.
6. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64**:29–63.
7. **Campo, G. M., S. Campo, A. M. Feriazzo, R. Vinci, and A. Calatroni.** 2001. Improved high-performance liquid chromatographic method to estimate aminosugars and its application to glycosaminoglycan determination in plasma and serum. J. Chromatogr. B **765**:151–160.
8. **Chatterjee, D., G. O. Aspinall, and P. J. Brennan.** 1987. The presence of novel glucuronic acid-containing, type-specific glycolipid antigens within *Mycobacterium* spp. Revision of earlier structures. J. Biol. Chem. **262**:3528–3533.
9. **Chatterjee, D., and K. H. Khoo.** 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. Cell. Mol. Life Sci. **58**: 2018–2042.
10. **Daffe, M., and P. Draper.** 1998. The envelope layers of mycobacteria with reference to their pathogenicity. Adv. Microb. Physiol. **39**:131–203.
11. **Danelishvili, L., M. Wu, B. Stang, M. Harriff, S. Cirillo, J. Cirillo, R. Bildfell, B. Arbogast, and L. E. Bermudez.** 2007. Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. Proc. Natl. Acad. Sci. USA **104**:11038–11043.
12. **Eckstein, T. M., J. T. Belisle, and J. M. Inamine.** 2003. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in *Mycobacterium avium* serovar 2. Microbiology **149**:2797–2807.
13. **Eckstein, T. M., J. M. Inamine, M. L. Lambert, and J. T. Belisle.** 2000. A genetic mechanism for deletion of the *ser2* gene cluster and formation of rough morphological variants of *Mycobacterium avium*. J. Bacteriol. **182**: 6177–6182.
14. **Eckstein, T. M., F. S. Silbaq, D. Chatterjee, N. J. Kelly, P. J. Brennan, and J. T. Belisle.** 1998. Identification and recombinant expression of a *Mycobacterium avium* rhamnosyltransferase gene (*rtfA*) involved in glycopeptidolipid biosynthesis. J. Bacteriol. **180**:5567–5573.
15. **Enomoto, K., S. Oka, N. Fujiwara, T. Okamoto, Y. Okuda, R. Maekura, T. Kuroki, and I. Yano.** 1998. Rapid serodiagnosis of *Mycobacterium avium-intracellulare* complex infection by ELISA with cord factor (trehalose 6, 6′-dimycolate), and serotyping using the glycopeptidolipid antigen. Microbiol. Immunol. **42**:689–696.
16. **Falkinham, J. O., III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9**:177–215.
17. **Field, S. K., D. Fisher, and R. L. Cowie.** 2004. *Mycobacterium avium* complex pulmonary disease in patients without HIV infection. Chest **126**:566–581.
18. **Fujiwara, N., N. Nakata, S. Maeda, T. Naka, M. Doe, I. Yano, and K. Kobayashi.** 2007. Structural characterization of a specific glycopeptidolipid containing a novel *N*-acyl-deoxy sugar from *Mycobacterium intracellulare* serotype 7 and genetic analysis of its glycosylation pathway. J. Bacteriol. **189**:1099–1108.
19. **Gerwig, G. J., J. P. Kamerling, and J. F. G. Vliegenthart.** 1978. Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary G.L.C. Carbohydr. Res. **62**:349–357.
20. **Hakomori, S.** 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**:205–208.
21. **Heidelberg, T., and O. R. Martin.** 2004. Synthesis of the glycopeptidolipid of *Mycobacterium avium* serovar 4: first example of a fully synthetic C-mycoside GPL. J. Org. Chem. **69**:2290–2301.
22. **Howard, S. T., E. Rhoades, J. Recht, X. Pang, A. Alsup, R. Kolter, C. R. Lyons, and T. F. Byrd.** 2006. Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. Microbiology **152**:1581–1590.
23. **Kaufmann, S. H.** 2001. How can immunology contribute to the control of tuberculosis? Nat. Rev. Immunol. **1**:20–30.
24. **Khoo, K. H., D. Chatterjee, A. Dell, H. R. Morris, P. J. Brennan, and P. Draper.** 1996. Novel *O*-methylated terminal glucuronic acid characterizes the polar glycopeptidolipids of *Mycobacterium habana* strain TMC 5135. J. Biol. Chem. **271**:12333–12342.
25. **Khoo, K. H., E. Jarboe, A. Barker, J. Torrelles, C. W. Kuo, and D. Chatterjee.** 1999. Altered expression profile of the surface glycopeptidolipids in drug-resistant clinical isolates of *Mycobacterium avium* complex. J. Biol. Chem. **274**:9778–9785.
26. **Kitada, S., R. Maekura, N. Toyoshima, N. Fujiwara, I. Yano, T. Ogura, M. Ito, and K. Kobayashi.** 2002. Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. Clin. Infect. Dis. **35**:1328–1335.
27. **Kitada, S., Y. Nishiuchi, T. Hiraga, N. Naka, H. Hashimoto, K. Yoshimura, K. Miki, M. Miki, M. Motone, T. Fujikawa, K. Kobayashi, I. Yano, and R. Maekura.** 2007. Serological test and chest computed tomography findings in patients with *Mycobacterium avium* complex lung disease. Eur. Respir. J. **29**:1217–1223.
28. **Krzywinska, E., and J. S. Schorey.** 2003. Characterization of genetic differences between *Mycobacterium avium* subsp. avium strains of diverse virulence with a focus on the glycopeptidolipid biosynthesis cluster. Vet. Microbiol. **91**:249–264.
29. **Maekura, R., Y. Okuda, A. Hirotani, S. Kitada, T. Hiraga, K. Yoshimura, I. Yano, K. Kobayashi, and M. Ito.** 2005. Clinical and prognostic importance of serotyping *Mycobacterium avium-Mycobacterium intracellulare* complex isolates in human immunodeficiency virus-negative patients. J. Clin. Microbiol. **43**:3150–3158.
30. **Marras, T. K., and C. L. Daley.** 2002. Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin. Chest Med. **23**:553–567.
31. **Maslow, J. N., V. R. Irani, S. H. Lee, T. M. Eckstein, J. M. Inamine, and J. T. Belisle.** 2003. Biosynthetic specificity of the rhamnosyltransferase gene of *Mycobacterium avium* serovar 2 as determined by allelic exchange mutagenesis. Microbiology **149**:3193–3202.
32. **McClatchy, J. K.** 1981. The seroagglutination test in the study of nontuberculous mycobacteria. Rev. Infect. Dis. **3**:867–870.
33. **McCloskey, J. A.** 1969. Mass spectrometry, p. 402. *In* J. M. Lowenstein (ed.), Methods in enzymology: lipid, vol. 14. Academic Press, New York, NY.
34. **McNeil, M., H. Gaylord, and P. J. Brennan.** 1988. *N*-formylkansosaminyl-(1-3)-2-*O*-methyl-D-rhamnopyranose: the type-specific determinant of serovariant 14 of the *Mycobacterium avium* complex. Carbohydr. Res. **177**:185–198.
35. **McNeil, M., A. Y. Tsang, and P. J. Brennan.** 1987. Structure and antigenicity of the specific oligosaccharide hapten from the glycopeptidolipid antigen of *Mycobacterium avium* serotype 4, the dominant *Mycobacterium* isolated from patients with acquired immune deficiency syndrome. J. Biol. Chem. **262**: 2630–2635.
36. **Miyamoto, Y., T. Mukai, N. Nakata, Y. Maeda, M. Kai, T. Naka, I. Yano, and M. Makino.** 2006. Identification and characterization of the genes involved in glycosylation pathways of mycobacterial glycopeptidolipid biosynthesis. J. Bacteriol. **188**:86–95.
37. **Odham, G., and E. Stenhagen.** 1972. Fatty acids, p. 211–228. *In* G. R. Waller (ed.), Biochemical application of mass spectrometry. Wiley-Interscience, New York, NY.
38. **Patterson, J. H., M. J. McConville, R. E. Haites, R. L. Coppel, and H. Billman-Jacobe.** 2000. Identification of a methyltransferase from *Mycobacterium smegmatis* involved in glycopeptidolipid synthesis. J. Biol. Chem. **275**:24900–24906.
39. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht.** 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. **36**:762–771.
40. **Tsang, A. Y., J. C. Denner, P. J. Brennan, and J. K. McClatchy.** 1992. Clinical and epidemiological importance of typing of *Mycobacterium avium* complex isolates. J. Clin. Microbiol. **30**:479–484.
41. **Wayne, L. G., and H. A. Sramek.** 1992. Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin. Microbiol. Rev. **5**:1–25.
42. **Woods, A., and J. R. Couchman.** 2001. Proteoglycan isolation and analysis, p. 10.7.1–10.7.19. *In* J. S. Bonifacino, M. Dasso, J. B. Harford, J. Lippincott-Schwartz, and K. M. Yamada (ed.), Current protocols in cell biology. Wiley Interscience, Hoboken, NJ.

Jpn.J.Leprosy 77. 29-36(2008)

Leprosy situation in Vietnam - reduced burden of stigma

Pham Dang Bang*[1, 2], Koichi Suzuki[3], Norihisa Ishii[3] and Tran Hau Khang[1, 2]

1)Dermatology Department, Hanoi Medical University, Vietnam
2)National Institute of Dermato-Venereology, Vietnam
3)Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, Japan

[Received / Accepted: 28 May, 2007]

Key words : elimination, leprosy, stigma, Vietnam, WHO

1. Background

a. Country profile

Vietnam, officially the Socialist Republic of Vietnam, is the easternmost nation on the Indochina Peninsula. It borders China to the north, Laos to the northwest, and Cambodia to the southwest. On the country's east coast lies the South China Sea. The capital of Vietnam is Hanoi and the largest and most populous city is Ho Chi Minh City. Vietnam, with its 4-level administrative system, is divided into 59 provinces and 5 province-level cities, which are further subdivided into districts and municipalities. Each district or municipality consists of 10-20 communes. Often, the Vietnamese government groups the various provinces into eight regions: Northwest, Northeast, Red River Delta, North Central Coast, South Central Coast, Central Highland, Southeast and Mekong River Delta. Vietnam extends approximately 331,688 square km in area. With a population of over 85 million, Vietnam is the 13th most populous country in the world.

b. Leprosy control system

Vietnam's National Leprosy Control Programme was established in 1982. The Leprosy control system had been intergrated into the Health care system which follows the Administrative system (Figure 1). The National Institute of Dermato-Venereology (NIDV) is leading institute responsible to the Ministry of Health (MOH) for skin diseases, sexually transmitted infections (STIs) and leprosy control in the whole country. In each province, there is a Dermato-Venereology Clinic, vertically under the NIDV, covering these three fields at provincial level. Dermato-Venereological activities including leprology work are integrated into general health system at district level. At the district's Social Diseases Unit in endemic zones, several practitioners are specially trained to work exclusively in leprosy field whereas in less endemic district, they are in charge of some contagious diseases such as leprosy, tuberculosis, malaria, HIV/AIDS, etc. In each commune that includes 2-5

*Corresponding author :
Pham Dang Bang. National Institute of Dermato-Venereology, Phuong Mai Street, Dong Da District, Hanoi 10000, Vietnam.
TEL : +84 - 4 - 576 - 4000 FAX : +84 - 4 - 576 - 1649
E-mail :bangphd@fpt.vn

villages with 1,000-3,000 people, leprosy as well as other social diseases are managed by one or two health workers. Because of poor infrastructure at Communal Health Stations, their main work is to refer suspect cases for diagnosis confirmation, to treat and follow up confirmed cases. Antileprosy drugs are stored at any levels, from central to local.

2. Leprosy control activities in the period 1975 - 2006

Dermato-Venereology profession network was established in 1975, immediately after the country's reunion and antileprosy was one of its main responsibilities. A patchy strategy was initially adopted for leprosy elimination activities due to lack of human and financial resources. A total of 21 special projects such as Leprosy Elimination Campaigns (LEC) and Special Action Projects for Elimination of Leprosy (SAPEL) were implemented from 1975, covering more than 2.1 million inhabitants. The projects led to the detection of 1920 new cases and helped clear some leprosy epidemicity pockets, mainly in the central highland and some southern provinces. Because leprosy activities did not cover the whole country, no exact data (prevalence, incidence, etc) was recorded at national level before 1982 but estimated prevalence rate of lep-

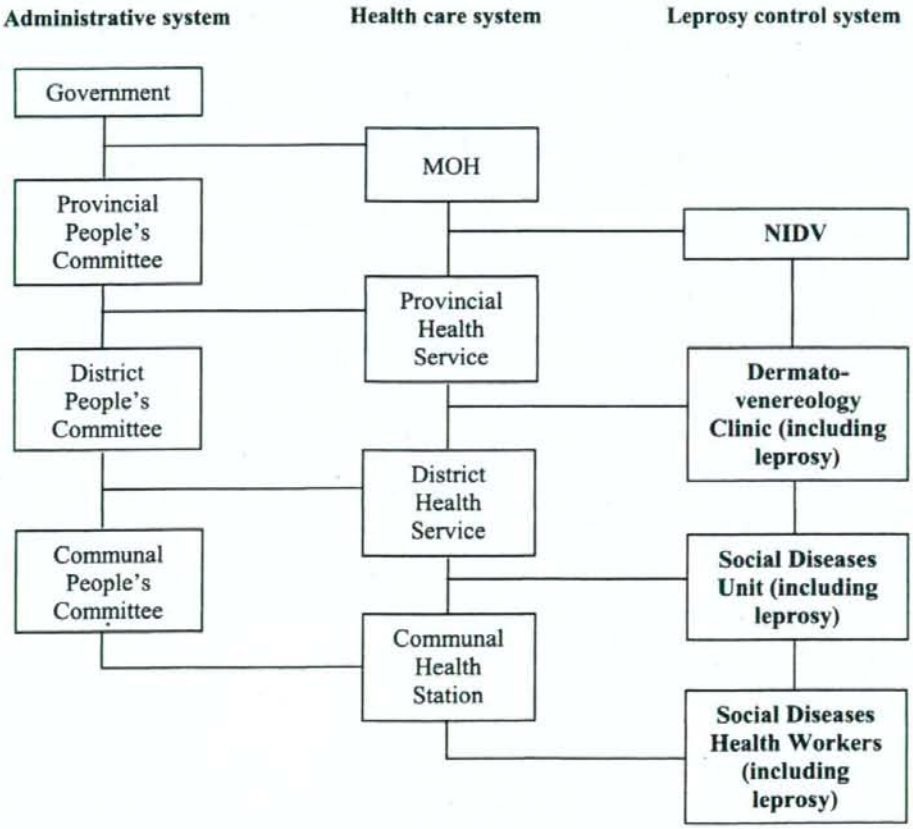

Figure 1. Leprosy control system according to health care and administrative system

rosy was 6 - 7/10,000 population in 1975. Most of patients moved into leprosaria and leprosy villages despite of the fact that there was no segregation law. MDT implementation started in 1983 resulting in dramatic reduction of prevalence rate with nearly 30,000 patients had completed treatment by the year 1995. In early 1990s, leprosy control system was reformed and strengthened with effective supports from international bodies. Thank to effective antileprosy activities, WHO's elimination goal (prevalence rate < 1/10,000 population) was achieved at national level in 1995 (Figure 2) and Vietnam launched its own elimination target of leprosy which are stricter that those were brought out by WHO, including 4 criteria:

- Prevalence rate is less than 0.2/10,000 population in 3 consecutive years
- Case detection rate is less than 1/100,000 population at the time of surveillance
- Grade 2 disability proportion among new cases is less than 15 %
- 20% of community leaders, health workers and high school pupils are randomly chosen for an interview; all of them have basic knowledge on leprosy.

In order to achieve these criteria, following solutions have been carried out:

a. Strengthening the leprosy control network from central to local levels

- extending antileprosy network to all provinces, including provinces with low prevalence rate
- building one referral center for each province or several nearing provinces
- building 3 leprosy and dermatology regional hospitals which are responsible for confirming difficult cases, managing persistent leprosy reactions, doing reconstructive surgery...
- holding retraining courses for leprosy control staff including practitioners, technicians, nurses and village volunteers
- promulgating legislative documents to encourage people working in leprosy field

b. Improving community awareness with IEC (Information, Education and Communication) activities

IEC, as experiences of some countries, plays a

Year	Prevalence rate (1/10,000)
1995	0.70
1996	0.68
1997	0.61
1998	0.44
1999	0.27
2000	0.23
2001	0.20
2002	0.16
2003	0.15
2004	0.10
2005	0.08
2006	0.07

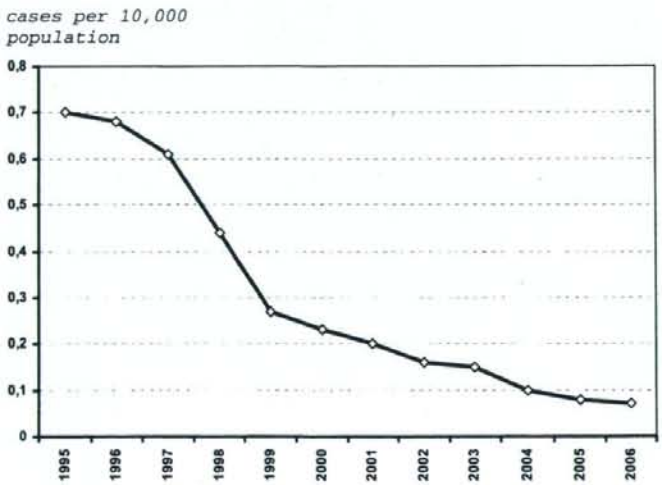

Figure 2. Prevalence rate of leprosy (1995 - 2006)

key role on stigma reduction in the community. Therefore, reasonable proportion of resources has been used for these activities. Priority population of IEC activities included community leaders and young people.

c. Strengthening case detection and management

- New case detection is always an important indicator of the programme. Good community awareness encouraged people with suspect signs and symptoms to go to the specialist for diagnosis confirmation. Other kinds of examinations are also carried out including contact examination, integrated examination, group examination...

In addition, SAPEL and LEC are also implemented in high endemic zones.

- All detected patients were treated with MDT free of charge. The duration of treatment for PB patients was 6 month with observed monthly dose of rifampicin and daily dose dapsone. For MB patient observed monthly dose of rifampicin and clofazimine was combined with daily dose of clofazimin and dapsone. The duration of treatment for MB patients used to be 24 months and then shortened to 12 months from 2005. The MDT storage was available at all communal health stations, even to all the village in the endemic zones.
- Handbooks were distributed to the patients, giving them useful guides to early detect the complications including, drug eruption, neuritis and other sign effects. All patients with these problems were referred to leprosy specialists for diagnosis.

d. Disabilities prevention and rehabilitation

Due to lack of financial resources, these activities were only under attention from 2000. Patients were taught to prevent themselves from deformities through handbooks and training courses. 80% of patients were provided with special shoes to prevent foot injury. 50% of the patients with eye closure problem were provided with special glasses. More than 1,000 patients received reconstructive surgery annually.

e. Conducting post-elimination surveillance project

A pilot post-elimination surveillance project based on protocol developed by WPRO has been operating in selected 40 provinces since 2002. The project has proven to be very effective for the leprosy control programme. Many training courses on leprosy field have been held for the health care workers at various levels. Provincial referral centers have been established, where many suspected cases have been to referred to confirm the diagnosis

f. Receiving national and international aids

The national leprosy control programme gained active support of national and international organizations, *i.e.* DFB, GLRA, NLR, AIFO, SMHF and Unilever Vietnam. Each year, national leprosy control programme received about 15 billions VND from the government including medicine, medical equipments and training fees.

3. Major achievements of the programme from 1995 - 2006

The significant achievements in reducing the burden of leprosy over twelve years were the result of success in implementing the mentioned solutions.

- Between 1995 and the end of 2006, about 100 millions participations were examined for leprosy detection
- Social stigma was removed, patients were treated at their home and they could work together with other community members. Their children can go

to school.

- WHO's elimination goal was reached at all provinces at the end of 2000.
- 19,429 new cases detected (of whom 12,099 were MB and 7,330 were PB) (Figure 5)
- Prevalence rate dropped from 0.70/10,000 population in 1995 to 0.07/10,000 population in 2006 (Figure 2)
- Child proportion among new cases reduced from 8.57% in 1995 to 5.56% in 2006 (Figure 6)
- Detection rate declined from 3.45/100,000 population in 1995 to 0.79/100,000 population in 2006 (Figure 3)
- Grade 2 disability rate among new cases decreased from 30.50% in 1995 to 17.27% in 2006 (Figure 4)
- Nearly 24,000 patients completed MDT with the completion rate was 99% (Figure 7)
- About 16,000 patients received rehabilitation activities

Year	No of new cases	Rate (1/100,000)
1995	2591	3.45
1996	2883	3.83
1997	2808	3.65
1998	2162	2.74
1999	1795	2.35
2000	1477	1.94
2001	1336	1.73
2002	1158	1.44
2003	940	1.18
2004	858	1.04
2005	746	0.90
2006	666	0.79

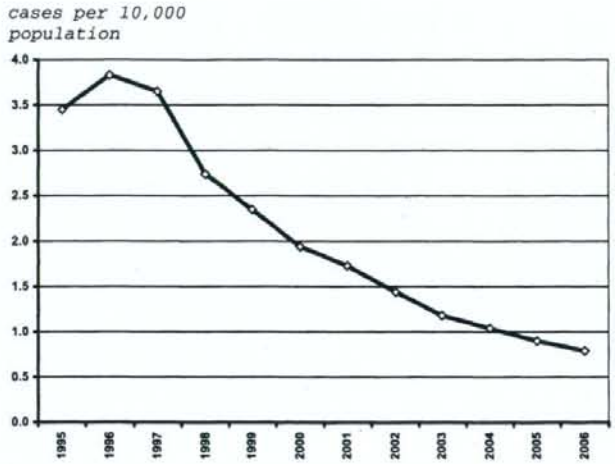

Figure 3. Detection rate of leprosy (1995-2006)

Year	No of new cases	Cases with grade II disability	Rate (%)
1995	2591	789	30.45
1996	2883	909	31.53
1997	2808	854	30.41
1998	2162	626	28.95
1999	1795	450	25.07
2000	1477	309	20.92
2001	1336	267	19.99
2002	1158	225	19.43
2003	949	179	18.86
2004	858	145	16.90
2005	746	121	16.22
2006	666	115	17.27

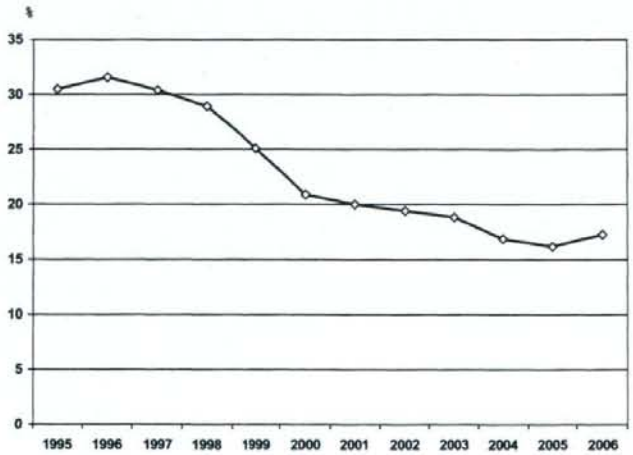

Figure 4. Grade II disability proportion among new cases (1995-2006)

4. Reduced stigma of leprosy in Vietnam

Leprosy has affected people in Vietnam at least hundreds of years ago. There were scattered writings on leprosy and leprosy affected patients in old documents. In the past, stigma towards leprosy patients and their families had adversely affected their quality of life due to its impacts on their mobility, interpersonal relationships, marriage, job finding, leisure and other social activities. All of these occurred in the community despite the fact that no segregation or discrimination laws has been promulgated to date.

At that time, people believed that leprosy had bad origin and was the punishment for sins the patients committed in the previous incarnation. In some communities, there is a belief that leprosy was hereditary so leprosy patients were forced to be infertilized.

Since National leprosy control programme was established, many activities have been done to re-

Year	No of new cases	No of MB cases	Rate (%)
1995	2591	1690	65.23
1996	2883	1807	62.68
1997	2808	1798	64.03
1998	2162	1278	59.11
1999	1795	1071	59.67
2000	1477	905	61.27
2001	1336	822	61.53
2002	1158	715	61.74
2003	949	590	62.17
2004	858	560	65.27
2005	746	452	60.59
2006	666	411	61.71

Figure 5. MB proportion among new cases (1995-2006)

Year	No of new cases	Caes with age < 15	Rate (%)
1995	2591	222	8.57
1996	2883	211	7.32
1997	2808	159	5.66
1998	2162	162	7.49
1999	1795	124	6.91
2000	1477	105	7.11
2001	1336	77	5.76
2002	1158	65	5.61
2003	949	52	5.48
2004	858	47	5.48
2005	746	47	6.30
2006	666	37	5.56

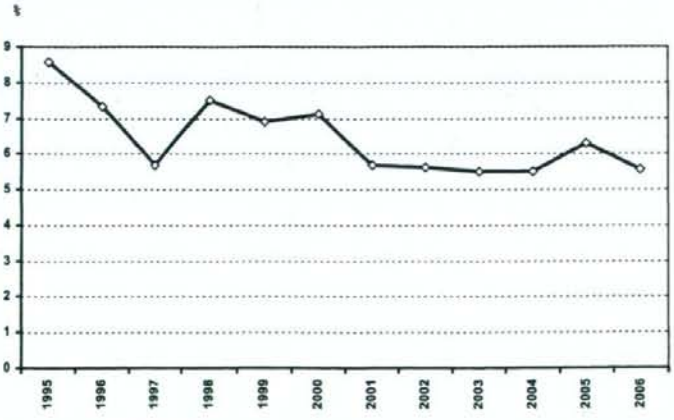

Figure 6. Children proportion among new cases (1995-2006)

duce the stigma in the community. The implementation of MDT, which effectively treated leprosy patients, itself eased the sigma burden by reducing visible deformity rate. The old words indicating leprosy such as "*cùi*" in the south part or "*hùi*" in the north part of the country have no longer been used because they imply something evil, degrading or immoral. Instead, the more scientific and natural term "*phong*" has been used to call the disease. In addition, higher knowledge of the curability and difficult-to-contamination of leprosy has been popularized and lead to more positive attitudes towards person affected with leprosy. The main features of leprosy are taught in the elementary school in the endemic areas.

Misconceptions about the causes of leprosy, that could have perpetuated stigma, were also prevalent among health care workers in the past. But the misconceptions have been gradually reduced due to the retraining courses held by the National Programme which provided health care workers with more accurate knowledge of leprosy. Moreover, the antileprosy work, since last decade, has been integrated into the general health care system, so that people recognized that leprosy is actually an infectious disease and deformed persons by leprosy are just like other handicapped person.

As a result of intensive activities, leprosy patients are now hardly affected by stigma from the society. Patients are treated free of charge with MDT at their own home and they can have a job suitable to their health. Marriage is accepted for leprosy patients and many patients have children after diagnosis. Public and private schools allow children born to leprosy patients to attend, and these children can study with other children in the community.

5. Post-elimination challenges

Even WHO's elimination goal was reached at all provinces at the end of 2000, relatively high case detection rate was still found in some area particularly in the central highland and some southern provinces. In 2006, there were 20 provinces reporting detection rate more than 1/100,000 population. The patients were not distributed equally but there were many pockets of patients that need more attention from the programme.

At the end of 2006, only 32 provinces reached

Year	PB	MB	Total
1995	1011	2449	3460
1996	1046	1785	2831
1997	981	1688	2669
1998	972	2257	3229
1999	839	2221	3060
2000	610	1210	1820
2001	568	910	1478
2002	515	847	1362
2003	331	711	1042
2004	555	654	1209
2005	534	409	943
2006	401	367	768

Figure 7. Number of patients complete MDT (1995-2006)

the Vietnam's 4 elimination criteria and 5 provinces still had prevalence rate more than 0.2/10,000 population. MB and child proportion were high, 61.71% and 5.56% respectively, indicating high infectiveness.

In the areas where prevalence rate is low, the health staffs in charge of leprosy have to do many other activities with different topics and there are few chances for them to see the lesions of active leprosy. As the result, their knowledge and practices regarding leprosy may be lost gradually.

6. Post-elimination activities

Based on the effectiveness of the post-elimination leprosy surveillance, the rest 24 provinces need to be covered by activities of the project. In the next period, the objects of the project are:

- sustaining the network of leprosy control programme despite the fact that low prevalence and detection rates in selected provinces
- conducting retraining courses on leprosy for the health staff at provincial, district and communal levels
- detecting all hidden cases of leprosy in the community
- sustaining and strengthening the awareness of leprosy in the community with IEC activities

7. Future requiremental aids

In the future, community directed activities must be carried out to maintain the achievements. In addition, high technologies on leprology are to be developed in central and regional institutes such as sequencing to detect drug resistance of mycobacteria; serology and other tests to make prognosis in high risk groups. Aids from developed countries are the important part of this plan.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Research on Emerging and Reemerging Infectious Diseases from The Ministry of Health, Labor, and Welfare of Japan and an International Cooperation Research Grant, Bureau of International Cooperation, International Medical Center of Japan (IMCJ) to N.I.

RESEARCH LETTER

Expression of adipose differentiation-related protein (ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*

Kazunari Tanigawa[1], Koichi Suzuki[1], Kazuaki Nakamura[1], Takeshi Akama[1], Akira Kawashima[1], Huhehasi Wu[1], Moyuru Hayashi[1], Shin-Ichiro Takahashi[2], Shoichiro Ikuyama[3], Tetsuhide Ito[4] & Norihisa Ishii[1]

[1]Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, Higashimurayama, Tokyo, Japan; [2]Laboratory of Cell Regulation, Department of Applied Animal Science, Graduate School of Agriculture and Life Science, The University of Tokyo, Yayoi, Tokyo, Japan; [3]Department of Immunobiology and Neuroscience, Division of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Tsurumihara, Beppu, Japan; and [4]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Maidashi, Fukuoka, Japan

Correspondence: Koichi Suzuki, Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan. Tel.: +81 42 391 8211; fax: +81 42 394 9092; e-mail: koichis@nih.go.jp

Received 23 June 2008; accepted 3 September 2008.
First published online 13 October 2008.

DOI:10.1111/j.1574-6968.2008.01369.x

Editor: Masao Mitsuyama

Keywords
Mycobacterium leprae; leprosy; lipid; adipose differentiation-related protein; perilipin.

Abstract

Mycobacterium leprae survives and replicates within a lipid droplet stored in the enlarged phagosome of histiocytes, a typical feature of lepromatous leprosy that is thought to be an important nutrient source for the bacillus. However, the underlying mechanisms by which lipids accumulate within phagosomes remain unclear. Recently, it was revealed that the lipid droplet-associated proteins, including ADRP and perilipin, play essential roles in lipid accumulation in adipocytes or macrophages. Therefore, we attempted to examine the role of these proteins in leprosy pathogenesis. ADRP and perilipin localized to the phagosomal membrane, which contains *M. leprae* in skin biopsy specimens of lepromatous leprosy. ADRP expression was transiently increased after phagocytosis in THP-1 cells. However, high levels of ADRP expression persisted only when live *M. leprae*, but not dead bacilli or latex beads, was added. Furthermore, although peptidoglycan, a Toll-like receptor 2 ligand, suppressed the expression levels of ADRP and perilipin, *M. leprae* infection inhibited this suppression. These results suggest that live *M. leprae* has the ability to actively induce and support ADRP/perilipin expression to facilitate the accumulation of lipids within the phagosome and to further maintain a suitable environment for the intracellular survival within the macrophage.

Introduction

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, shows a broad spectrum of clinical manifestation. Lepromatous leprosy is characterized by widespread skin lesions consisting of unrestricted multiplication of bacilli inside foamy histiocytes due to an impaired cellular immune response. In these lesions, *M. leprae* lives and replicates in a foamy or an enlarged phagosome within macrophages that are filled with lipids.

However, it is unclear how such a large amount of lipids is recruited and accumulated in phagosomes containing *M. leprae*. It is thought that *M. leprae* survives by utilizing the lipids and fatty acids as carbon source in the granuloma environment, where the oxygen tension gradient is relatively low (Chan *et al.*, 1989). Therefore, it is important to understand the mechanisms by which lipid droplets accumulate within the phagosome in order to better understand the strategy that *M. leprae* uses to survive within host cells.

Recent studies have highlighted the important role of proteins that mediate lipid accumulation in cells. In animal cells, these include the structurally related members of the PAT protein family, which is named after perilipin, adipophilin/adipose differentiation-related protein (ADRP), and the tail-interacting protein of 47 kDa (TIP47) (Greenberg *et al.*, 1993; Blanchette-Mackie *et al.*, 1995; Servetnick *et al.*, 1995; Brasaemle *et al.*, 1997a, b; Wolins *et al.*, 2001; Miura *et al.*, 2002). ADRP is a ubiquitously expressed PAT family

protein that serves as a scaffolding during lipid droplet formation. The protein has fatty acid-binding properties and stimulates fatty acid uptake in cells (Gao *et al.*, 2000). Overexpression of ADRP increased triglyceride accumulation, and knockdown of ADRP by a specific small interfering RNA decreased the pool of cytosolic lipid droplets (Magnusson *et al.*, 2006). ADRP expression has been suggested for use as a sensitive marker of lipid loading in human blood monocytes and in human monocyte-derived macrophages incubated with oxidized low-density lipoproteins (LDL) (Llorente-Cortes *et al.*, 2007). In contrast, perilipin was originally described as a lipid droplet-associated protein expressed only in adipocytes (Serlachius & Andersson, 2004), but it has been identified recently in other tissues including the vascular wall, where expression was demonstrated in macrophages and smooth muscle cells (Forcheron *et al.*, 2005). Studies on perilipin null mice suggest that perilipin shields lipid droplets from hormone-sensitive lipase activity under basal conditions and is necessary for cyclic adenosine monophosphate-stimulated triglyceride lipolysis (Sztalryd *et al.*, 2003; Miyoshi *et al.*, 2006). Perilipin has also been found on the surface of lipid droplets in lipid-loaded human-cultured THP-1 monocytes (Persson *et al.*, 2007), and the protein is expressed in primary human macrophages incubated with acetylated LDL. Perilipin expression increased over time in cell culture when human monocytes spontaneously differentiated into macrophages (Persson *et al.*, 2007). Furthermore, perilipin content has been correlated with lipid content in foam cells (Larigauderie *et al.*, 2006).

To date, it is not known whether ADRP/perilipin play roles in lipid accumulation in lepromatous leprosy, a typical example of intracellular parasitization of bacteria. In this study, we examine the expression and localization pattern of ADRP/perilipin both *in vivo* and *in vitro* and explore the impact of *M. leprae* on these cellular activities.

Materials and methods

Acid-fast staining and immunohistochemistry

Archived formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical staining as described previously (Suzuki *et al.*, 1998a, 2006b). Briefly, deparaffinized sections were incubated with anti-ADRP antibody (PROGEN Biotechnik GmbH, Heidelberg, Germany) diluted to 1 : 200 or anti-perilipin antibody (Affinity BioReagents, Golden, CO) diluted to 1 : 100 for 1 h at room temperature. Slides were washed with Dulbecco's phosphate-buffered saline containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20). The peroxidase-labeled streptavidin–biotin method using the LSAB2 Kit (DAKO, Carpinteria, CA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) was used according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 2006b). Sections were then stained with carbol fuchsin and counterstained with methylene blue to visualize acid-fast mycobacteria. Archived formalin-fixed, paraffin-embedded tissues were used according to the guidelines approved by the National Institute of Infectious Diseases (Tokyo, Japan).

Cell culture and infection with *M. leprae*

THP-1, a human promonocytic cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in 10 cm tissue culture dishes in RPMI medium supplemented with 10% charcoal-treated fetal bovine serum, 2% nonessential amino acids, and 50 mg mL^{-1} penicillin/streptomycin at 37 °C in 5% CO_2. *Mycobacterium leprae* was prepared from the footpads of nude mice as described previously (Suzuki *et al.*, 2006a, b). Live or heat-killed (80 °C, 30 min) bacilli (3×10^7) or latex beads (Fluoresbrite microspheres; Technochemical, Tokyo, Japan) were added to 3×10^6 cells, multiplicity of infection (MOI) 10. Cells were further cultured for RNA and protein purification.

RNA preparation, reverse transcriptase (RT)-PCR and quantitative real-time PCR

RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) as described previously (Suzuki *et al.*, 1999a, b, 2006a). RNA preparation from skin smear samples was performed as follows: Stainless-steel blades (Feather Safety Razor Co. Ltd, Osaka, Japan) used to obtain slit-skin smear specimens were rinsed in 1 mL of sterile 70% ethanol and centrifuged at 20 000 *g* for 1 min at 4 °C. RNA was isolated from pellets with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), using the same protocol as that used for cultured cells. RNA was eluted in 20 μL of elution buffer and treated with 0.1 U μL^{-1} of DNase I (TaKaRa, Kyoto, Japan) at 37 °C for 1 h in order to degrade any contaminating genomic DNA. RNA concentration and purity were assessed using a Genequant Pro Spectrophotometer (GE Healthcare UK Ltd, Buckinghamshire, UK). Total RNA from each sample was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) as described by Suzuki & Kohn (2006) and Suzuki *et al.* (2006a). The following primers were used to amplify cDNA: 5′-TGTGGAGAAGAC CAAGTCTGTG-3′ (ADRP forward) and 5′-GCTTCTGAAC CAGATCAAATCC-3′ (ADRP reverse); 5′-GCTCTGATTCT ATGGCTTGGTT-3′ (perilipin forward) and 5′-TGTGTCA AAACCTTCTGTCTGG-3′ (perilipin reverse); and 5′- AGC CATGTACGTAGCCATCC-3′ (actin forward) and 5′-TGTG GTGGTGAAGCTGTAGC-3′ (actin reverse). Touchdown PCR was performed using a Thermal Cycler Dice (Takara)

as described previously (Suzuki *et al.*, 1998a, b, 2006a). The products were analyzed by 2% agarose gel electrophoresis. Skin smear samples were taken after obtaining written informed consent.

Real-time PCR was carried out in a solution containing 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), 50 nM of each primer, and cDNA template. The same primers that were used for RT-PCR analysis were utilized. Samples were analyzed using an ABI Prism 7000 analyzer (Applied Biosystems) with an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analyzed using ABI PRISM 7000 SDS Software Version 1.1 (Applied Biosystems). All samples were amplified in triplicate from the same RNA preparation, and the experiment was repeated three times.

Protein preparation and Western blot analysis

Cellular protein was extracted and analyzed as described previously (Suzuki *et al.*, 1999a, b, 2002). Briefly, cells were lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 20% glycerol, and protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Basel, Switzerland) for 1 h. After centrifugation, the supernatant was transferred and 20 μg of protein was used for Western blotting. Samples were heated in sodium dodecyl sulfate sample loading buffer at 95 °C for 5 min and loaded on a polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was washed with PBST (PBS with 0.1% Tween 20), placed in blocking buffer (PBST containing 5% nonfat milk) overnight, and then incubated with anti-ADRP (1 : 1000) or anti-perilipin (1 : 1000) antibody. After washing with PBST, the membrane was incubated for 1 h with biotinylated donkey anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) and streptavidin–HRP (Amersham Bioscience), according to the manufacturer's protocol, and developed using ECL Plus reagent (Amersham Biosciences).

Transient transfection and luciferase assay

A luciferase reporter plasmid, p5 × NF-κB-luc, was purchased from Stratagene (La Jolla, CA). Transient transfection was conducted using FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 1999a, b). THP-1 cells were incubated for 36 h after transfection, after which peptidoglycan (2 μg mL^{-1} final concentration) or *M. leprae* (MOI 10) was added. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol (Suzuki *et al.*, 1998a, b, 1999a, b).

Other reagents

All other reagents were purchased from Sigma Aldrich (Saint Louis, MO).

Statistical analysis

Results are expressed as mean ± SE. Student's *t*-test was used for statistical analysis. P values < 0.05 were regarded as statistically significant.

Results

ADRP and perilipin are expressed in skin lesions of lepromatous leprosy

ADRP and perilipin expression was assayed in skin biopsy specimens taken from patients with lepromatous leprosy. Immunohistochemical staining was used to visualize ADRP and perilipin, and *M. leprae* was demonstrated using acid-fast staining on the same section (double staining). The immunoreactivity of ADRP and perilipin was observed in the majority of foamy histiocytes that contain acid-fast bacilli (i.e. *M. leprae*) (Fig. 1a and b, respectively). At a higher magnification, both were clearly observed on the membranes of phagosomes containing *M. leprae* (Fig. 1c and d). The staining was also observed in old lesions of lepromatous leprosy where bacilli were primarily degenerative or not visible (Fig. 1e and f), which is similar to the case of CORO1A localization (Suzuki *et al.*, 2006b). However, expression of ADRP and perilipin was not detected in the granulomatous lesions of tuberculoid leprosy where *M. leprae* is not usually detected (Fig. 1g and h). RT-PCR of the skin smear specimens confirmed the presence of ADRP/perilipin mRNA in all of the samples tested, although the expression levels were variable among samples (Fig. 2). These results indicate that ADRP/perilipin is expressed in the lesions of lepromatous leprosy and localizes to the phagosomal membrane of histiocytes that contain *M. leprae*.

ADRP/perilipin expression is induced by *M. leprae* infection in THP-1 cells

We next examined ADRP/perilipin expression in human THP-1 cells following *M. leprae* infection. RT-PCR analysis revealed a rapid increase in ADRP mRNA 1 h after infection and in perilipin mRNA 6 h after infection, which was maintained for 12 h postinfection (Fig. 3a). The increase in mRNA levels was more prominent when a larger number of bacilli (MOI 100) were used (data not shown). Western blot analysis demonstrated a significant increase in ADRP protein as early as 3–6 h after infection and within 9–12 h for perilipin protein (Fig. 3b). Immunocytochemistry showed that both ADRP and perilipin localized to the phagosomal membrane of THP-1 cells containing *M. leprae* (Fig. 3c and d,

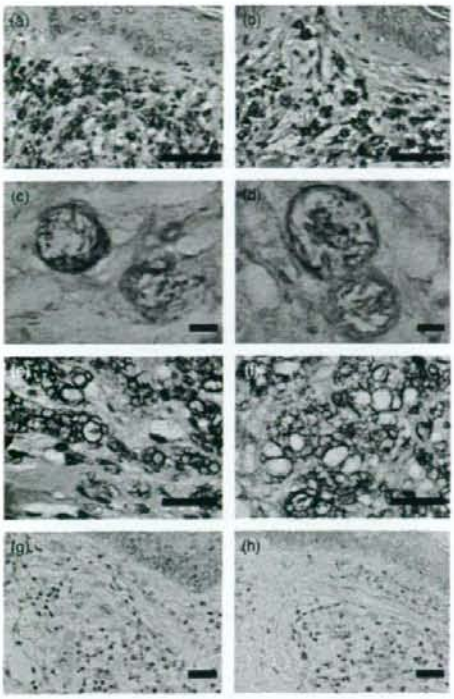

Fig. 1. Localization of ADRP and perilipin in skin lesions of lepromatous leprosy. Formalin-fixed, paraffin-embedded tissue sections from fresh (a, b, c, and d) and old (e and f) lesions from lepromatous leprosy and from tuberculoid leprosy (g and h) were immunostained for ADRP and perilipin (brown coloration), followed by acid-fast staining (pink coloration) and methylene blue or hematoxylin counterstaining. Photomicrographs of ADRP (a, c, e, and g) and perilipin (b, d, f, and h) immunostaining are shown. Original magnification: × 200 (a, b, g, and h; scale bar = 25 μm); × 1000 (c and d; scale bar = 5 μm); × 400 (e and f; scale bar = 25 μm).

respectively). Immunofluorescence staining confirmed the results (data not shown).

To clarify whether the observed increase of ADRP/perilipin is specific for *M. leprae* or is instead nonspecific for phagocytosis, we compared the effect of introducing live *M. leprae*, heat-killed *M. leprae*, or latex beads. In all three cases, a similar increase in ADRP/perilipin mRNA expression was observed by RT-PCR (Fig. 4a) and quantitative real-time PCR in 6 h (Fig. 4b and c). Interestingly, however, ADRP expression remained at a high level for 72 h only in cultures to which live *M. leprae* was added (Fig. 4a and b). Perilipin mRNA reverted to original levels in 72 h in all the cases (Fig. 4a and c). Correspondingly, the transient expression of ADRP protein levels in 6 h and its prolonged expression following live *M. leprae* infection were confirmed

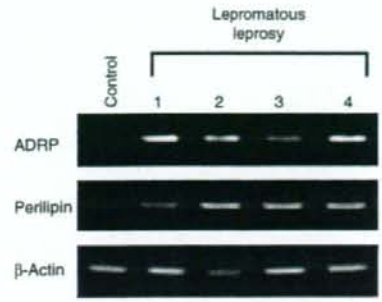

Fig. 2. Detection of ADRP and perilipin mRNA in skin smear samples. RNA was isolated from skin smear specimens taken from four patients with lepromatous leprosy. After treatment with DNase, RT-PCR was performed. The control sample was obtained from skin granuloma where mycobacteria were not found.

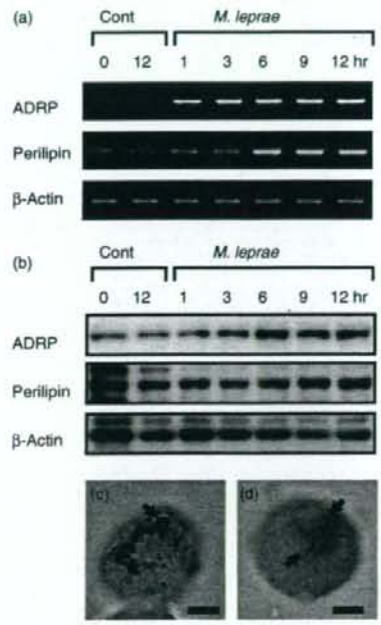

Fig. 3. Induction of ADRP and perilipin expression by *Mycobacterium leprae* infection of THP-1 cells. THP-1 cells (3×10^6) were cultured in a six-well plate and infected with 3×10^7 cells of *M. leprae*. After incubating for the indicated time, total RNA and total cellular protein were purified and RT-PCR analysis (a) and Western blot analysis (b) were performed. THP-1 cells grown on glass cover slips were infected with *M. leprae* and subjected to ADRP (c) and perilipin (d) immunocytochemistry. Arrows indicate a positive signal around phagosomes. Original magnification: × 1000. Scale bar = 3 μm.

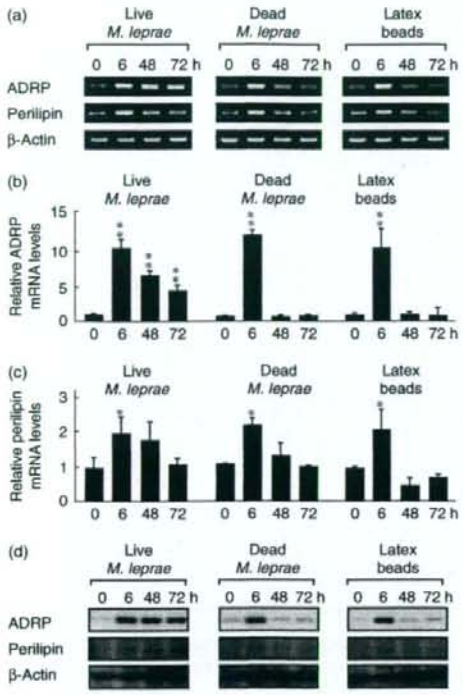

Fig. 4. Only live *Mycobacterium leprae* induces expression of ADRP. THP-1 cells (3×10^6) were cultured in a six-well plate and infected with 3×10^7 cells of live *M. leprae*, heat-killed (80 °C for 30 min) *M. leprae* or latex beads. After incubating for the indicated time, total RNA was purified and RT-PCR analysis (a) and quantitative real-time PCR (b and c) was performed. The results from real-time PCR were normalized with actin expression and expressed as a relative value against 0 h. Similarly, cellular protein was purified and Western blot analyses of ADRP and perilipin were performed (d). The graph shows the mean ± SD. One asterisk indicates a value of $P < 0.05$; two asterisks indicate a value of $P < 0.001$.

by Western blot analysis (Fig. 4d). The increase in perilipin protein levels was limited, as were the changes in mRNA levels. Because the phagosome is formed within a few hours after phagocytosis of mycobacteria (Ferrari *et al.*, 1999; Suzuki *et al.*, 2006b), these results suggest that transient expression of ADRP/perilipin is induced by phagocytosis; however, expression of ADRP is sustained by an unknown component(s) derived from live *M. leprae*.

Mycobacterium leprae infection reverses the effect of peptidoglycan on suppression of ADRP mRNA expression

It is well known that cell wall lipoproteins of mycobacteria stimulate Toll-like receptor 2 (TLR2) and activate a downstream signaling cascade (Jones *et al.*, 2001). Therefore, we first attempted to evaluate the effect of TLR-mediated signaling in *M. leprae*-infected macrophages. Phorbol myristate acetate (PMA)-treated THP-1 cells were used as a model of activated macrophages in which to evaluate the effect of peptidoglycan and *M. leprae*. The amount of peptidoglycan ($2\,\mu g\,mL^{-1}$) and *M. leprae* (MOI 10) used similarly induced NF-κB-dependent promoter activation (Fig. 5a), suggesting that both have a similar ability to stimulate TLR2. PMA strongly induced ADRP mRNA levels as reported (Wei *et al.*, 2005), but the effect was weak on perilipin mRNA levels (Fig. 5b and c). Peptidoglycan strongly reduced the ADRP mRNA levels and weakly reduced perilipin expression (Fig. 5d). Interestingly, *M. leprae* infection did not affect ADRP expression levels at all, and modulated perilipin expression only weakly (Fig. 5e). We then examined the effect of *M. leprae* on the ADRP/perilipin levels that were reduced by peptidoglycan. The addition of *M. leprae* reversed the suppressive effect of peptidoglycan, allowing continued expression of high levels of ADRP (Fig. 5f). The results of quantitative real-time PCR analysis of ADRP mRNA levels confirm these results (Fig. 5g). Perilipin mRNA levels were not significantly affected by *M. leprae* in PMA-activated THP-1 cells (Fig. 5e and f; real-time PCR data not shown). These results suggest that *M. leprae* infection inhibits TLR2-mediated suppression of ADRP expression.

Discussion

In this study, we demonstrated that ADRP/perilipin localizes to the phagosomal membrane of histiocytes, which contains numerous bacilli, in skin lesions of lepromatous leprosy. In addition, we showed that *M. leprae* infection increases expression of ADRP/perilipin mRNA and protein in THP-1 cells. These results suggest that *M. leprae* regulates ADRP/perilipin expression for the accumulation of lipid droplets that will be utilized as a nutrient for intracellular survival.

In fact, there is evidence that pathogenic mycobacteria primarily use fatty acids rather than carbohydrates as carbon substrates during infection. Respiration of *Mycobacterium tuberculosis* grown in mouse lungs is strongly stimulated by fatty acids but is unresponsive to carbohydrates (Zahoor *et al.*, 2005). Several glycolytic enzymes are apparently dispensable for growth and persistence of *M. tuberculosis* in mice (Mathur *et al.*, 2005), and the terminal step in glycolysis is blocked in the closely related zoonotic pathogen *Mycobacterium bovis* as a result of a mutation in *pykA*, which encodes pyruvate kinase (Keating *et al.*, 2005). Furthermore, persistence of *M. tuberculosis* in mice is facilitated by isocitrate lyase, an enzyme essential for the metabolism of fatty acids (Gould *et al.*, 2006). Therefore, it would be

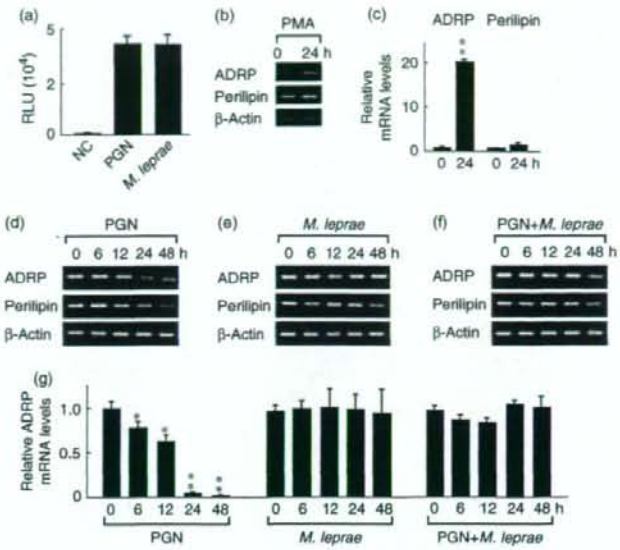

Fig. 5. *Mycobacterium leprae* inhibits the ability of peptidoglycan to suppress the expression of ADRP. TLR2 activation in THP-1 cells was assessed with a luciferase assay using an NF-κB-dependent reporter gene (a). THP-1 cells (3×10^6) were cultured in a six-well plate and treated with PMA at a final concentration of 20 ng mL^{-1} for 24 h. mRNA expression of ADRP and perilipin was assessed using RT-PCR (b) and quantitative real-time PCR (c). The results from real-time PCR were normalized with actin expression and reported as a relative value against 0 h. PMA-stimulated cells were treated with peptidoglycan (2 μg mL^{-1}) (d), *M. leprae* (MOI 10) (e), or both (f). After incubating for the indicated time, total RNA was purified and RT-PCR analysis was performed. Changes in ADRP mRNA levels were further evaluated by quantitative real-time PCR (g). The graph shows the mean ± SD. *, $P < 0.05$; **, $P < 0.001$.

plausible to speculate that *M. leprae* also utilizes fatty acids as carbon substrates within host cells.

Only live cells of *M. leprae* could sustain prolonged expression of ADRP/perilipin, while transient expression was induced by dead bacilli or latex beads. This situation is quite similar to the accumulation of CORO1A, also known as tryptophan aspartate-containing coat protein, on the phagosomal membrane, which results in inhibition of lysosomal fusion and accounts for the survival of bacilli (Ferrari *et al.*, 1999). CORO1A accumulates in the phagosomal membrane that contains *M. leprae* in lepromatous leprosy (Suzuki *et al.*, 2006b). Furthermore, only live, but not heat-killed, *M. bovis* Bacillus Calmette–Guérin could maintain CORO1A expression and localization on the phagosome (Ferrari *et al.*, 1999). Therefore, *M. leprae* might actively recruit ADRP/perilipin, as well as CORO1A, to the phagosomal membrane to create an appropriate and favorable environment within the phagosome. Although it is difficult to identify responsible component(s) maintaining ADRP expression because of the lack of an *in vitro* cultivation method of *M. leprae*, our results potentially suggest that *M. leprae* have an ability to stimulate ADRP expression as well as CORO1A expression. It appears that high expression levels of ADRP and perilipin were maintained in the clinical specimens (Figs 1 and 2), while expression decreased after several hours in cultured THP-1 cells (Fig. 4). This may be a reflection of a current limitation of leprosy research – *in vitro* cultivation of *M. leprae*, even in cultured cells, is not possible. Therefore, continuous stimulation of live *M. leprae* cannot be carried out for a long period of time *in vitro*. Whether other pathogenic and nonpathogenic mycobacteria have similar effects on ADRP/perilipin induction is an interesting issue for future study.

The changes in mRNA expression pattern and protein levels differ between ADRP and perilipin. It has been shown in adipocytes that the proteins that coat lipid droplets, which are determined by multiple factors, change during lipid droplet biogenesis (Ducharme & Bickel, 2008). Although the precise molecular mechanisms that regulate transcription of ADRP and perilipin in macrophages have not been resolved, our results suggest the differential roles of two PAT proteins in *M. leprae*-infected macrophages.

TLR2 also localizes to phagosomal membranes that contain *M. leprae* (Suzuki *et al.*, 2006b). In the present study, peptidoglycan, a TLR2 ligand, suppressed ADRP/perilipin expression in macrophages. It is known that bacterial cell wall components bind TLR2 and stimulate downstream signaling cascades. This signal activates the expression of proinflammatory cytokines, chemokines and type I interferons in order to launch innate immunity as a first line of defense against infection (Underhill *et al.*, 1999). Thus, based on the present finding, we speculate that activation of innate immunity results in suppression of ADRP/perilipin, which in turn reduces lipid accumulation within the infected macrophage and accounts for the nutritional diminution for the intracellular pathogen.

We also showed that *M. leprae* inhibits TLR2-mediated suppression of ADRP expression. Because both peptidoglycan

and *M. leprae* induce similar activation of NF-κB under the conditions used, the underlying molecular mechanism by which *M. leprae* exerts such an opposite effect is unknown. It is speculated that *M. leprae* activates a hitherto unrecognized TLR-independent pathway that results in inhibition of TLR-mediated ADRP/perilipin suppression. Such a function would further contribute to the creation of a lipid-rich environment that is favorable for survival of the pathogen.

In conclusion, we have identified a mechanism that may contribute to the lipid accumulation observed in the foamy histiocytes of lepromatous leprosy. *Mycobacterium leprae* induces and actively sustains ADRP expression. Further studies determining the detailed mechanisms by which lipids accumulate in the *M. leprae*-infected phagosome will provide a better understanding of leprosy pathogenesis. It will also provide insights that may lead to the development of a new therapeutic method that inhibits the expression and/or the localization of ADRP and perilipin.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sport, Science and Technology of Japan (to K.S.) and by a Grant-in-Aid for Research on Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan (to N.I.). The authors thank M. Mishima, D.B. Pham, S. Aizawa, and S. Sekimura (LRC, NIID) for helpful discussions.

References

Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondinone CM, Theodorakis JL, Greenberg AS & Londos C (1995) Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J Lipid Res* **36**: 1211–1226.

Brasaemle DL, Barber T, Kimmel AR & Londos C (1997a) Post-translational regulation of perilipin expression. Stabilization by stored intracellular neutral lipids. *J Biol Chem* **272**: 9378–9387.

Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ & Londos C (1997b) Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res* **38**: 2249–2263.

Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, Sibille JC, Snapper M, Aisen P & Bloom BR (1989) Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci USA* **86**: 2453–2457.

Ducharme NA & Bickel PE. (2008) Lipid droplets in lipogenesis and lipolysis. *Endocrinology* **149**: 942–949.

Ferrari G, Langen H, Naito M & Pieters J (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* **97**: 435–447.

Forcheron F, Legedz L, Chinetti G, Feugier P, Letexier D, Bricca G & Beylot M (2005) Genes of cholesterol metabolism in human atheroma: overexpression of perilipin and genes promoting cholesterol storage and repression of ABCA1 expression. *Arterioscl Thromb Vas* **25**: 1711–1717.

Gao J, Ye H & Serrero G (2000) Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. *J Cell Physiol* **182**: 297–302.

Gould TA, van de Langemheen H, Munoz-Elias EJ, McKinney JD & Sacchettini JC (2006) Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*. *Mol Microbiol* **61**: 940–947.

Greenberg AS, Egan JJ, Wek SA, Moos MC Jr, Londos C & Kimmel AR (1993) Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proc Natl Acad Sci USA* **90**: 12035–12039.

Jones BW, Heldwein KA, Means TK, Saukkonen JJ & Fenton MJ (2001) Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages. *Ann Rheum Dis* **60**: (suppl 3): iii6–iii12.

Keating LA, Wheeler PR, Mansoor H, Inwald JK, Dale J, Hewinson RG & Gordon SV (2005) The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for *in vivo* growth. *Mol Microbiol* **56**: 163–174.

Larigauderie G, Bouhlel MA, Furman C, Jaye M, Fruchart JC & Rouis M (2006) Perilipin, a potential substitute for adipophilin in triglyceride storage in human macrophages. *Atherosclerosis* **189**: 142–148.

Llorente-Cortes V, Royo T, Juan-Babot O & Badimon L (2007) Adipocyte differentiation-related protein is induced by LRP1-mediated aggregated LDL internalization in human vascular smooth muscle cells and macrophages. *J Lipid Res* **48**: 2133–2140.

Magnusson B, Asp L, Boström P, Ruiz M, Stillemark-Billton P, Lindén D, Borén J & Olofsson SO (2006) Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arterioscl Thromb Vas* **26**: 1566–1571.

Mathur D, Ahsan Z, Tiwari M & Garg LC (2005) Biochemical characterization of recombinant phosphoglucose isomerase of Mycobacterium tuberculosis. *Biochem Bioph Res Co* **337**: 626–632.

Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B & Kimmel AR (2002) Functional conservation for lipid storage droplet association among perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *J Biol Chem* **277**: 32253–32257.

Miyoshi H, Souza SC, Zhang HH *et al.* (2006) Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *J Biol Chem* **281**: 15837–15844.