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Aggregation of mycobacteria caused by disruption of fibronectin-attachment protein-encoding gene

Yuji Miyamoto, Tetsu Mukai *, Fumihiko Takeshita, Noboru Nakata, Yumi Maeda, Masanori Kai, Masahiko Makino

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan

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Abstract

The fibronectin-attachment protein (FAP) is conserved among several species of mycobacteria. Although this protein is associated with attachment and internalization of bacteria to host cells via fibronectin, the physiological role of the protein still remains unclear. To investigate this point, we generated FAP gene disruptant in *Mycobacterium smegmatis*. The gene disruption, verified by Southern blot and PCR analysis, induced changes on the bacteria, which are associated with strong aggregation and alteration of cell surface properties. Increased hydrophobicity and Congo red accumulation was observed in the FAP gene disruptant. In addition, the complementation experiment demonstrated that the corresponding gene restored wild type morphology in the disruptant. These results indicate that the FAP affects the cell surface properties, and its deletion lead to enhanced aggregation of the *M. smegmatis*.

Keywords: Fibronectin-attachment protein; Gene disruption; *Mycobacterium smegmatis*

1. Introduction

Mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are the causative agents of human disease. Bacterial attachment to host cells is important in the process of infection, and fibronectin-attachment protein (FAP), a family of fibronectin-binding [1–6], is one of the representative bacterial components involved in the attachment. FAP was initially isolated from *Mycobacterium vaccae* culture and was subsequently shown to have the ability to bind to fibronectin [2]. The binding of FAP to fibronectin enhanced the bacterial binding affinity and subsequent internalization of mycobacteria to host cells. In fact, the treatment of *M. leprae* with anti-FAP antibody inhibited its binding to peripheral nerve Schwann cells [3]. On the other hand, the FAP of *Mycobacterium bovis* BCG is thought to be capable of inducing cellular immunity, and the FAP of *Mycobacterium avium* induced strong T-cell response in mice [7,8]. Therefore, the fibronectin-binding activity of FAP is one of factors determining the mycobacterial virulence.

The mycobacterial cell wall is reported to possess the activities to protect mycobacteria from various bacteri-ocidal actions, including host immune system and anti-mycobacterial chemotherapeutic agents [9]. The deletion of the polyacyltrehalose and glycopeptidolipids (GPLs) induced the morphological changes that may be involved in the induction of alternative host immune responses against bacteria [10,11]. In other cases, the morphological changes such as bacterial aggregation consequently triggered the biogenesis of phagolysozome in human neutrophils, while the declumped single cells possessed no ability to trigger it [12]. However, in spite

* Corresponding author. Tel.: +81-42-391-8211; fax: +81-42-394-9092.

E-mail address: tmukai@nih.go.jp (T. Mukai).

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of the fact that FAP is a cell wall component of mycobacteria, it has not been clarified yet whether gene disruption of FAP induce the morphological or functional changes as lipid component do. A gene disruption is a well-established method to elucidate the direct role of each gene. Therefore, in this study, we generated FAP-deficient mutant of mycobacteria using *M. smegmatis* as a model bacteria and characterized the properties of the mutant to reveal physiological role of the FAP.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used as host strains for cloning experiments. Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco laboratories) with 0.05% Tween 80 or Middlebrook 7H10 agar with 0.5% glycerol, both supplemented with 10% ADC enrichment (Difco laboratories) and kanamycin (25 μg/ml).

2.2. DNA techniques

Plasmids used and constructed in this study are listed in Table 1. Genomic DNA for polymerase chain reaction (PCR) and Southern blot analysis was isolated from mycobacterial strains as previously described [16]. Transformations of *M. smegmatis* strains were carried out by electroporation with a Bio-Rad Gene Pulsar (Bio-Rad) set at 1.3 V, 25 μF, and 200 Ω as described by Parish and Stoker [17]. Sequences of the oligonucleotide primers used for PCR are shown in Table 2. Probes for

Table 1
Bacterial strains and plasmids used in this study

	Characteristics	Source/reference
Bacteria		
E. coli	DH5α; cloning host	
M. smegmatis	mc^2 155; wild type	[13]
	MF96; *fapS*-disruptant	This study
M. leprae	Thai-53	
M. bovis BCG	Pasteur (ATCC35734)	
M. avium	JATA51-01 (ATCC25291)	
Plasmid		
pUC19	*E. coli* cloning vector	
pBluescript II SK (+)	*E. coli* cloning vector	
pMV261	*E. coli Mycobacterium* shuttle vector carrying *hsp60* promoter cassette	[14]
pMV306kan	A site-specific integrating mycobacterial vector	[15]
pUDFAP	pUC19 with a 3.0 kb *Hind* III–*Xba* I fragment (upstream) and a 3.0 kb *Xba* I–*Kpn* I fragment (downstream)	This study
pUDFAPKm	pUDFAP with a 1.1 kb *Hind* III fragment (kanamycin resistant cassette)	This study
pFAPS	pMV306kan with a 1.5 kb *Xba* I–*Nhe* I fragment (FAP-S-expression cassette)	This study
pFAPL	pMV306kan with a 1.5 kb *Xba* I–*Nhe* I fragment (FAP-L-expression cassette)	This study
pFAPB	pMV306kan with a 1.5 kb *Xba* I–*Nhe* I fragment (FAP-B-expression cassette)	This study
pFAPA	pMV306kan with a 1.5 kb *Xba* I–*Nhe* I fragment (FAP-A-expression cassette)	This study

Table 2
Oligonucleotide primers used in this study

Primer	Sequences[a]	Restriction site
US1	5′-CCC AAG CTTTAC CTT GAC CCG GCC CGC GC-3′	*Hind* III
UA1	5′-GCTCT AGA CGG TCA CCG CAG CCA GCG TC-3′	*Xba* I
DS1	5′-GCTCT AGA CCG ATG CGC CGC CGG AGA TGA-3′	*Xba* I
DA1	5′-GGGGT ACC GCA GGT CCA TCT CGT CGC GC-3′	*Kpn* I
U1	5′-CGTGG CGG TCC GGG CCT CGT CG-3′	
D1	5′-CGGGC GCT CTC GGC TTC GGC GG-3′	
S1	5′-CCCAAG CTT ATA TGT ACG AGT CGG AC TCG ATG-3′	*Hind* III
S2	5′-CCATC GAT ATC AGG CCG GAG GCA TCA TCT CC-3′	*Cla* I
B1	5′-CGGGA TCC CAT GCA TCA GGT GGA CCC CAA C-3′	*Bam*H I
B2	5′-GGAA TTC TCA GGC CGG TAA GGT CCG CTG-3′	*Eco*R I
L1	5′-CGGGA TCC CAT GAA TCA GGT TGA CCT GGA C-3′	*Bam*H I
L2	5′-AACTG CAG CTA TCC AAC AGG TGC CGG AGC-3′	*Pst* I
A1	5′-GGAA TTC ATGG ATC AGG TGG AAG CGA C-3′	*Eco*R I
A2	5′-CCATC GATATC AGG CCG AGA GGG TCT GCT G-3′	*Cla* I

[a] Underlined indicates restriction site.

Southern blot were prepared by using a Roche DIG labelling kit (Roche) according to the instructions.

2.3. Construction of suicide vector containing fapS-disrupted sequences

To generate the non-polar mutation in the gene encoding FAP of *M. smegmatis* (named as *fapS*), suicide vector containing DNA fragment flanking the *fapS* gene and kanamycin resistance cassette was constructed. A 3.0 kb fragments both upstream and downstream to *fapS* gene was amplified from *M. smegmatis* mc^2155 genomic DNA using the following two pairs of primers, for upstream; US1 (including *Hind* III site) and UA1 (including *Xba* I site); for downstream DS1 (including *Xba*I site) and DA1 (including *Kpn* I site). The PCR products were digested with restriction enzymes and cloned into *Hind* III–*Xba* I and *Xba* I–*Kpn* I site of pUC19 to give pUDFAP. The kanamycin resistance cassette, a selective marker of integration, was excised from pMV261 with *Hind* III and *Spe* I and cloned into corresponding sites of pBluescript II SK (+). The resulting plasmid was digested with *Kpn* I and *Xba* I, and derived fragment was cloned into the corresponding sites of pUC19. The 1.1 kb *Hind* III fragment harboring kanamycin resistance cassette was then cloned into pUDFAP to create the plasmid, pUDFAPKm, carrying the disrupted sequences.

2.4. Generation of the fapS-disrupted strain

The two-step recombination was conducted to disrupt the *fapS* gene located on the *M. smegmatis* chromosome. The suicide vector pUDFAPKm was introduced into the *M. smegmatis* mc^2155 by electroporation, and single crossover strains (plasmid-integrated transformants) were selected on the 7H10 plate containing 25 μg/ml kanamycin. A kanamycin-resistant colony was subjected to repeated cultivation in the 7H9 medium without kanamycin to excise the pUC19 region and kanamycin resistant cassette through the second crossover event. After several subcultures, the kanamycin-sensitive clones were selected and their genomic DNA was subjected to Southern blot and PCR analysis using the primers U1 and D1 to confirm the disruption of the *fapS* gene.

2.5. Construction of the FAP expression vectors for complementation

The *fapS* (FAP-S) gene and its homologous gene of FAP-B (*M. bovis* BCG), FAP-L (*M. leprae*) and FAP-A (*M. avium*) were used to construct the expression vector for complementation of *fapS*-mutant. Four genes were amplified from each genomic DNA with following primers; S1 (including *Hind* III site and start codon) and S2 (including *Cla* I site and stop codon) for *fapS*, B1 (including *Bam*H I site and start codon) and B2 (including *Eco*R I site and stop codon) for FAP-B, L1 (including *Bam*H I site and start codon) and L2 (including *Pst* I site and stop codon) for FAP-L, and A1 (including *Eco*R I site and start codon) and A2 (including *Cla* I site and stop codon) for FAP-A. The PCR products were digested with each restriction enzyme and cloned into the corresponding sites of pMV261. The expression cassette, consisting of *hsp60* promoter, the FAP gene and transcriptional terminator, were excised with *Xba* I and *Nhe* I and inserted into *Xba*I site of pMV306kan (kindly gifted from Dr. W.R. Jacobs, Jr.), a site-specific integrating mycobacterial vector, to give complementation vector pFAPS, pFAPB, pFAPL and pFAPA, respectively. These vectors were introduced into the *fapS* mutant by electroporation. Transformants were selected on the 7H10 plate containing 25 μg/ml kanamycin.

2.6. Morphological observation

The wild type, mutant strain and its complemented strains were cultured for two days in 2 ml of Middlebrook 7H9 broth. Aliquots of cultured broth were stained by Ziehl-Neelsen method and the bacteria was morphologically examined under light microscopy BX60 (Olympus). The images were acquired using a microscope digital camera DP50 (Olympus) and Viewfinder Lite software.

2.7. Assessment of cell surface hydrophobicity

The hydrophobicity of the bacteria was assessed by measuring the adherence of bacteria to hydrocarbons, *n*-hexadecane and xylene, as previously described with some modification [11,18]. Briefly, the 0.5 ml of *n*-hexadecane or xylene was added to the test tube containing 2.0 ml of phosphate buffered saline (PBS)-washed bacterial suspension, of which OD_{650} titer was adjusted at 1.0, and vigorously mixed using vortex for 1 min. After standing at room temperature for 20 min, the absorbance (OD_{650}) of the lower aqueous phase was measured. The hydrophobicity of bacteria was evaluated by calculating the reduction of absorbance values. The hydrophobic bacteria are assumed to remain in upper phase because of adhesion to hydrocarbons.

Congo red binding assay was also conducted to measure the bacterial hydrophobicity [11,19]. The bacteria was cultured for two days in 5 ml of Middlebrook 7H9 broth supplemented with the Congo red (100 μg/ml). The cells recovered from cultured broth were washed with PBS to eliminate unbound Congo red completely, and then resuspended in 0.2 ml of acetone. After gently shaking for 2 h, cells were pelleted by centrifugation and the concentration of Congo red released from bacteria into supernatant was measured spectrophotometrically

at 488 nm. The relative binding index was defined as the OD_{488} of acetone extract divided by OD_{650} of the original broth after certain period of cultivation.

2.8. Isolation and analysis of lipids

Total lipids were extracted from 50 ml of bacterial cell with $CHCl_3/CH_3OH$ (2:1, v/v) for several hours at room temperature. The lipid extracts from the organic phase were separated from the aqueous phase and evaporated to dryness. For analysis of GPLs, the lipids extracts were subjected to mild alkaline hydrolysis as previously described [20]. Total lipids and GPLs were analyzed by thin layer chromatography (TLC) on silica gel 60 plates (Merck) using $CHCl_3/CH_3OH/H_2O$ (30:8:1, v/v/v) and $CHCl_3/CH_3OH$ (9:1, v/v) as solvent respectively and visualized by spraying with a solution of 10% H_2SO_4 and subsequent charing.

2.9. Statistical analysis

Students' *t* test was applied to demonstrate statistically significant differences.

3. Results

3.1. Sequence analysis of fapS gene

The gene data base analysis of FAP-L (ML2055) and FAP-B (AF013569) indicated that the putative ORF of FAP, designated as *fapS*, was located between 1,810,474 and 1,811,535 bases of contig 3310 of the unfinished *M. smegmatis* genome database of in the Institute for Genomic Research (http://www.tigr.org). As shown in Fig. 1, the BLAST search analysis of the amino acids sequences of various FAP indicated that the homology of FAP-S with FAP-B (AF013569), FAP-A (U53585) and FAP-L (ML2055) was 51%, 48% and 47%, respectively. The homology was confirmed by FASTA program. We deduced the fibronectin-binding region of *M. smegmatis* FAP by homology analysis to that of *M. avium* and *M. bovis* BCG which were previously described [21].

3.2. Disruption of fapS gene

For the functional analysis of the FAP in *M. smegmatis*, the *fapS* gene was disrupted by the homologous recombination using the suicide vector. From the kanamycin-resistant isolates, MF96 was isolated as the putative mutant (Fig. 2). Southern blot analysis was performed on *Eco*R I-digested chromosomal DNA from wild type *M. smegmatis* and mutant strain MF96 to reveal the target gene disruption. Two different probes corresponding to the sequences in upstream (P1) and downstream (P2) of the *fapS* region were constructed (Fig. 2(a)). The probes P1 and P2 hybridized to the predicted 2.4 kb and 2.8 kb *Eco*RI fragments of the wild type strain, respectively, while both probes hybridized to sole 4.2 kb fragment in the strain MF96. The *fapS* gene disruption was further confirmed by using PCR analysis,

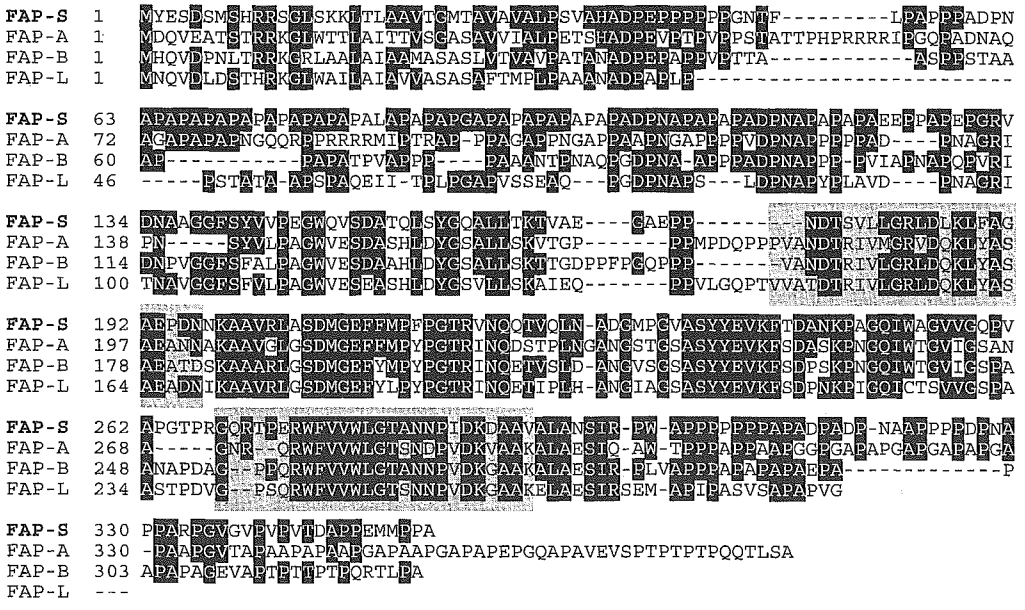

Fig. 1. Alignment of deduced amino acid sequences of *fapS* gene product (FAP-S) with FAP-A (*M. avium*), FAP-B (*M. bovis* BCG) and FAP-L (*M. leprae*). The amino acids identical to those of FAP-S are indicated by black boxes. The putative sequences of fibronectin-binding region are shown in gray background.

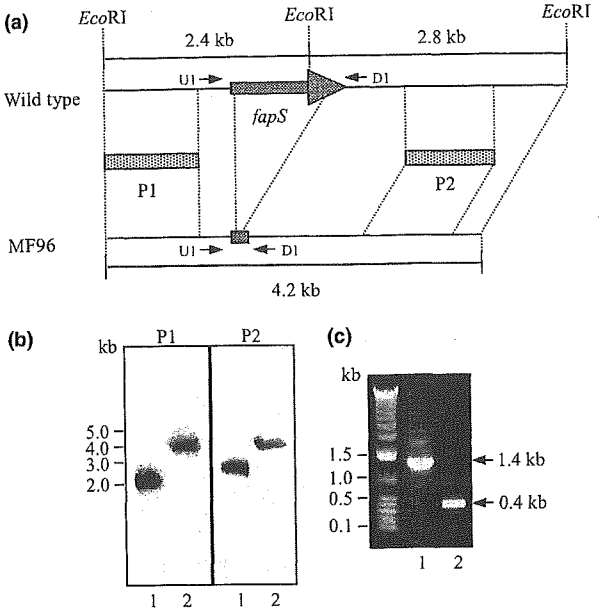

Fig. 2. Disruption of *fapS* gene. (a) Schematic diagram of the *fapS* region on the chromosome of wild type *M. smegmatis* (mc^{2}155) and the mutant strain MF96. The position of the two hybridization probes, P1 and P2, are also shown. The arrows indicate the primers (U1, D1) used for PCR analysis. (b) Southern blot analysis of wild type (lane 1) and strain MF96 (lane 2). Chromosomal DNA was digested with *Eco*R I and hybridized with probe P1 and P2. C, PCR analysis of wild type (lane 1) and the strain MF96 (lane 2) using the primers indicated above.

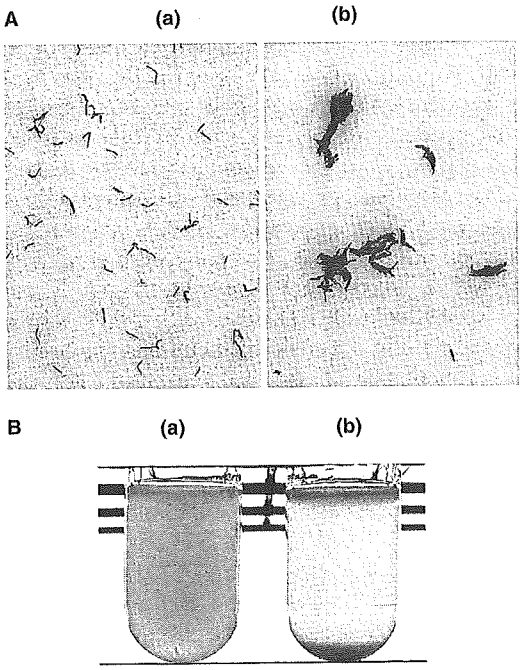

Fig. 3. Morphological observation of wild type (a) and mutant strain MF96 (b). (A) Light microscopic observation of cultured broth diluted 1:100 in PBS containing 0.2% skim milk followed by Ziehl-Neelsen staining. Magnification, 1000×. (B) Cultured broths left for 1 h without shaking.

in which the primers (U1 and D1) were constructed outside the *fapS* gene. As expected, while 1.4 kb fragment was amplified from wild type, 0.4 kb fragment was revealed by strain MF96. The size difference between the strains (1.0 kb) indicated that most of *fapS* gene was deleted.

3.3. *Morphology of* fapS *mutant*

The *fapS* mutant, cultured in liquid medium, showed a morphological change. Observation by the light microscopy revealed that the strain MF96 bacteria clumped together (Fig. 3A(b)), whereas most of wild type cells grew as a single cell and distributed equally in the field (Fig. 3A(a)). The strain MF96 cells quickly settled down to the bottom of the tube, whereas wild type remained dispersed in the broth (Fig. 3B). However, aggregation of strain MF96 was not attributable to the growth rate, moreover no differences were observed between MF96 and wild type strain in the colony morphology (data not shown). In order to confirm that the enhanced aggregation of strain MF96 was induced by the disruption of *fapS* gene, *fapS* gene was complementarily introduced into the *fapS* mutant (Fig. 4). The strain MF96 was transformed with the vectors containing each expression cassette of the FAP from

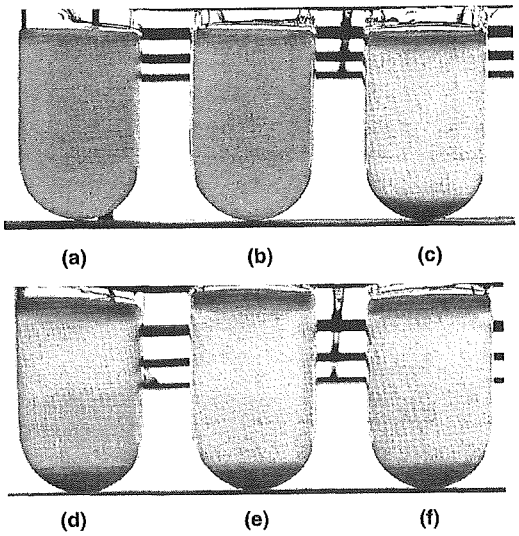

Fig. 4. Complementation experiment. Observation of wild type (a) and mutant strain MF96 transformed with pFAPS (b), pMV306kan (c), pFAPL (d), pFAPB (e) and pFAPA (f). All cultured broths were left for 1 h without shaking.

M. smegmatis, *M. leprae*, *M. bovis* BCG and *M. avium*, and their expressions in the complemented MF96 strains were confirmed by Western blot analysis of cell wall fractions obtained by disrupting the cell wall using

Mini-BeadBeater. The level of FAP expression was similar among the complemented strains (data not shown). The monoclonal Ab used in the Western blot solely reacted with cell wall fraction. The strain MF96 lost the property to aggregate each other by transformation with parent *M. smegmatis*-derived FAP gene (pFAPS). However, the strains transformed with other mycobacterial FAP expression vectors did not reverse the aggregation property, and they were similar to the strain MF96 which were transformed with a control vector (pMV306kan). Therefore, the FAP from other mycobacteria could not supplement the aggregation-inhibiting function of *M. smegmatis*-derived FAP, in spite of FAP genes being complementarily introduced. These results demonstrated that the bacterial aggregation induced in the strain MF96 was caused by the disruption of *fapS*.

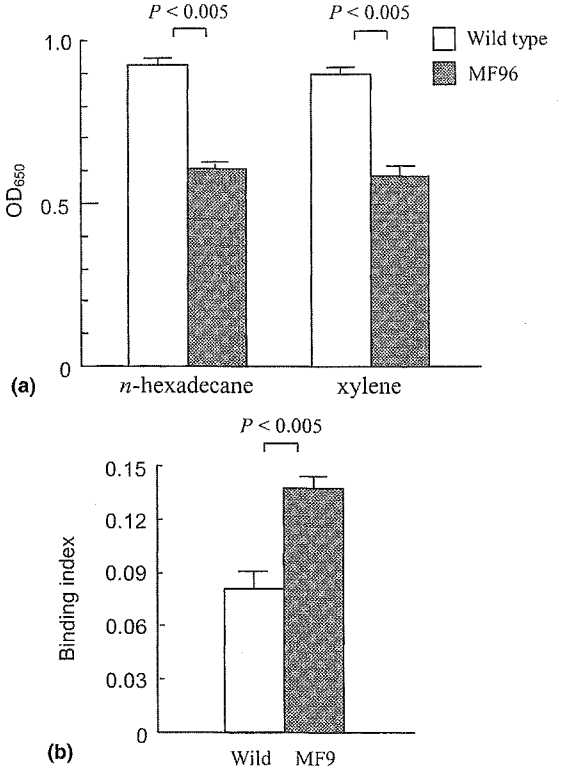

Fig. 5. (a) The cell surface hydrophobicity based on the degree of adherence to two hydrocarbons (*n*-hexadecane and xylene), assessed by the reduction in absorbance of aqueous phase separated from hydrocarbon mixture including cells of wild type (open square) and mutant strain MF96 (filled square). (b) Congo red binding of wild type (open square) and mutant strain MF96 (filled square). The binding index is derived from OD_{488} of acetone extract divided by OD_{650} of cultured broth. Representative of more than three separated experiments are shown. Assays were done in triplicate, and the results are expressed as means ± SD.

3.4. Cell surface properties of fapS mutant

It might be reasonable to speculate that *fapS* gene disruption induces the change of bacterial surface properties. Therefore, hydrophobicity and Congo red binding ability of strain MF96 were assessed. The hydrophobicity assay was basically assessed by measuring the degree of adherence of bacteria to hydrophobic molecules, such as *n*-hexadecane and xylene (Fig. 5(a)). The OD_{650} of aqueous phase in the strain MF96 was significantly lower than that in wild type *M. smegmatis*. Similar results were obtained in both assays using *n*-hexadecane and xylene (Fig. 5(a)). These results suggested that strain MF96 adhered more easily to hydrophobic molecule and remained in the upper hydrophobic phase. Furthermore, the hydrophobicity of strain MF96 was assessed using Congo red binding assay (Fig. 5(b)). The concentration of Congo red bound to bacterial surface and subsequently solubilized was significantly higher in strain MF96 than that in the wild type (Fig. 5(b)).

Next, the role of FAP in the bacterial attachment to host cells (J774 cells) was examined by Ziehl Neelsen staining method. There was no significant difference in the number of attached or intracellular bacteria between

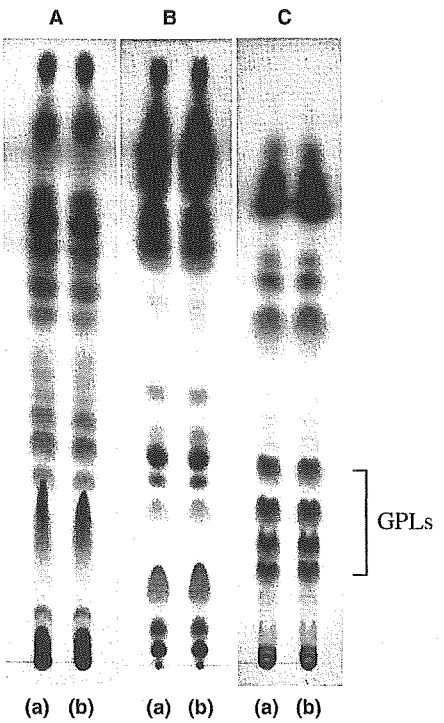

Fig. 6. Comparison of the lipid composition of wild type (a) and mutant strain MF96 (b). (A) Total lipids developed with $CHCl_3$/CH_3OH (9:1, v/v). (B) Total lipids developed with $CHCl_3$/CH_3OH/H_2O (30:8:1, v/v/v). (C) Total lipids subjected to mild alkaline hydrolysis and developed with $CHCl_3$/CH_3OH (9:1, v/v). GPLs, glycopeptidolipids.

the fibronectin-opsonized wild and MF96 mutant strains (data not shown).

3.5. Lipid analysis

In order to find out whether the *fap* gene disruption induces the alteration of cell wall lipid composition of *M. smegmatis* and causes aggregation of the mutant strain, TLC analysis using different solvent systems on total lipids from wild type and mutant strain was carried out. The TLC profiles of both strains were very similar to each other, and no detectable alterations were observed in the total lipid content as well as GPLs (Fig. 6).

4. Discussion

The FAP is involved in the attachment and internalization of mycobacteria to host cells. However, little is known about physiological role of FAP, such as how FAP is involved in bacterial interaction with the host cells. In this study, we undertook a gene disruption approach to construct the gene mutant of *M. smegmatis* to characterize the function of FAP

The FAP disruptant showed increased hydrophobic properties and a change in morphology which caused an enhanced bacterial aggregation. Previously, it has been reported that the absence or poor content of some glycolipids was induced by the reduction of biosynthetic enzyme activities and this caused an aggregation of bacteria along with the changes in the cell surface properties [10,11,22]. Also, the other family of fibronectin-binding protein, such as antigen 85 complex in *M. tuberculosis*, possess mycolyltransferase activity required for maintaining the integrity of the mycobacterial cell envelope [23–25]. However, comparative TLC analysis on total lipids extracts in different solvent system revealed no significant differences in the lipid profile between wild type and the FAP mutant (Fig. 6). Although the mycobacterial proteins, PE_PGRS and HBHA, are known to promote aggregation of cells [26,27], there is no report indicating that the absence of such a protein component would directly lead to the more aggregated phenotype. Thus, the FAP seems not to have influence on the biosynthesis of cell wall lipid, but show an enhanced aggregation of the *M. smegmatis*.

Analysis of the amino acid sequences of FAP showed that the hydrophobic amino acids, alanine and proline, are relatively rich compared to the other mycobacterial proteins [21,28], and these amino acids are notably present in the N-terminal region of FAP (Fig. 1). These facts may indicate that FAP is an amphiphilic molecule consisting of both hydrophobic and hydrophilic part similar to glycolipid. Alanine and proline rich region of FAP may correspond to acyl chains of glycolipid such as polyacyltrehalose and GPLs which are inserted into the hydrophobic core composing mycolic acid chain [10,11]. FAP mutant, generated by gene disruption, showed increased surface hydrophobicity as observed by enhanced adherence to hydrocarbons (Fig. 5). The increased hydrophobicity may be due to the exposure of hydrophobic lipid molecules which are otherwise located inside the cell wall in the wild type strain and FAP may have functioned as the amphiphilic layer with the hydrophilic proteins on the surface. Therefore, the increased hydrophobicity of the mutant could lead to the aggregation of bacteria.

The analysis of the deduced amino acid sequences of *fapS* gene product indicated that its homology with the FAP of other mycobacteria was around 50%, although the deduced fibronectin-binding region of the FAP was conserved in various mycobacteria including *M. avium*, *M. bovis* BCG and *M. leprae* (Fig. 1) [21,28]. The morphology of the bacteria was not recovered when the genes encoding FAP of *M. bovis* BCG, *M . leprae* and *M. avium* were introduced into the mutant strain, while the complementation with *M. smegmatis*-derived *fapS* reverse the aggregation property (Fig. 4). The possible explanation for non-recovery of the morphology of the bacteria is due to (1) low homology except the fibronectin-binding domain which is highly conserved between the FAP homologues, (2) interaction of FAP with other specific components in the cell wall of *M. smegmatis*. It has been reported that FAP locates in the cell wall fraction of mycobacteria [2]. However, its precise location in the cell wall is different in each mycobacteria [1,3,5]. In fact, in contrast to *M. bovis* BCG, the FAP of *M. smegmatis* does not appear on the bacterial surface as far as examined by fluorescence-activated cell sorting (FACS) using FAP monoclonal antibody which can detect the *M. smegmatis*-derived FAP by Western blot analysis (data not shown). This fact might be another reason why FAPs from other mycobacteria failed to reverse the aggregation property of MF96 mutant strain. Furthermore, it may be able to explain why there is no significant difference of host cell attachment activity between wild type and MF96 mutant strains.

Taken together, our study indicates that the FAP possesses novel function which are indispensable to prevent the bacterial aggregation that might contribute to the understanding of the mycobacterial biogenesis.

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Genotyping of *Mycobacterium leprae* on the Basis of the Polymorphism of TTC Repeats for Analysis of Leprosy Transmission

Masanori Matsuoka,[1]* Liangfen Zhang,[1] Teky Budiawan,[2] Keisuke Saeki,[3] and Shinzo Izumi[4]

Leprosy Research Center, National Institute of Infectious Diseases, Tokyo,[1] and National Leprosarium Oshima-Seisho-En, Kagawa,[3] Japan, and Leprosy-TB Program, Provincial Health Service, Manado, North Sulawesi,[2] and Leprosy Study Group, Tropical Disease Center, Kampus C UNAIR, Airlangga University, Surabaya,[4] Indonesia

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The polymorphism of TTC repeats in *Mycobacterium leprae* was examined using the bacilli obtained from residents in villages at North Maluku where *M. leprae* infections are highly endemic (as well as from patients at North Sulawesi of Indonesia) to elucidate the possible mode of leprosy transmission. TTC genotypes are stable for several generations of passages in nude mice footpads and, hence, are feasible for the genotyping of isolates and epidemiological analysis of leprosy transmission. It was found that bacilli with different TTC genotypes were distributed among residents at the same dwelling in villages in which leprosy is endemic and that some household contacts harbored bacilli with a different genotype from that harbored by the patient. Investigations of a father-and-son pair of patients indicated that infections of bacilli with 10 and 18 copies, respectively, had occurred. Genotypes of TTC repeats were found to differ between a son under treatment and two brothers. These results reveal the possibility that in addition to exposure via the presence of a leprosy patient with a multibacillary infection who was living with family members, there might have been some infectious sources to which the residents had been commonly exposed outside the dwellings. A limited discriminative capacity of the TTC polymorphism in the epidemiological analysis implies the need of searching other useful polymorphic loci for detailed subdivision of clinical isolates.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection. It has long being believed that the source of infection is untreated leprosy patients. It has also been predicted that multidrug therapy (MDT) with strong bactericidal antibiotics (such as rifampin) would reduce the source of infection and consequently interrupt further transmission to others. The number of new cases, however, has shown no substantial decline even though MDT with strong bactericidal drugs has been used in the past two decades. It is reported that about 600,000 to 700,000 new cases are continuously found in the world every year (20), which suggests that the transmission of leprosy bacilli still occurs, especially in countries of endemicity. Elucidation of the mode of transmission would be essential to help prevent new infection. The differentiation of strains of leprosy bacilli by genomic polymorphism might be of great value in efforts to understand the mode of transmission of the disease.

The range of molecular techniques for epidemiological analysis has expanded in recent years, and there are now many genotypic methods that allow a high level of discrimination between bacterial strains. Restriction fragment length polymorphism analysis, which is the method most widely used for molecular epidemiology of tuberculosis, is not applicable for leprosy, since *M. leprae* cannot be grown in artificial medium, and almost no divergence was found by this fingerprinting assay (19). Shin et al. discovered a genomic divergence of *M. leprae* by the variation of TTC repeats (17) and subdivided 34 isolates into 15 subtypes. Genotyping according to the TTC repeats for fragments amplified by PCR seemed to be feasible for molecular epidemiological analysis of leprosy transmission.

A previous study by Saeki et al. revealed that *M. leprae* existed on the surface of nasal cavities of residents in areas of endemicity (16). Here, we report the distribution of different TTC genotypes of *M. leprae* among family members of each dwelling in the villages with high-level leprosy prevalence and inconsistent genotypes obtained from patients and their family members in the same dwelling. The results strongly supported previously proposed hypotheses on the existence of an infectious source(s) other than that of patients living with family members.

* Corresponding author. Mailing address: Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, Tokyo, 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-394-9092. E-mail: matsuoka@nih.go.jp.

MATERIALS AND METHODS

Nasal swab samples from residents in villages where leprosy is endemic. Nasal swab samples which showed positive PCR results in a previous study by Saeki et al. that was designed to elucidate the distribution of *M. leprae* in the places where leprosy is endemic (16) were used in this study for the comparison of TTC genotypes among family members. These samples originated from residents in two Indonesian villages in North Maluku with a high-level prevalence of leprosy, Gamtala and Lolori. In Gamtala village, 353 nasal swab samples and 277 samples in Lolori village were collected. The population of Gamtala village was 509 (median age, 25) and that of Lolori was 434 (median age, 20); the numbers of houses were 105 and 77, respectively. The prevalence of leprosy in Gamtala was 4.0% (14/353), and the paucibacillary/multibacillary (PB/MB) case ratio was 10/4. In Lolori, the prevalence was 3.3% (9/277) and the PB/MB ratio was 9/0 (16). MDT recommended by the World Health Organization was introduced in the middle of the 1990s in these areas. No tap water was supplied, and several families shared one well for bathing, washing, and cooking. Living surroundings were almost the same in both villages. The surface of the nasal cavity was swabbed with sterilized wet cotton swabs (Mentip1P-75, Nippon Menbo, Tokyo, Japan), which were then frozen until use. The cotton was washed in 500 μl of phosphate-buffered saline containing 0.05% Tween 80 to release the sample from the cotton swab, and the aliquot was centrifuged at 10,000 × *g* for 20 min. The sediment was then digested with lysis buffer to prepare the template DNA (5) and subjected for sequencing as described below.

TABLE 1. Stability of TTC repeats of *M. leprae* strains during serial passage

Strain	Origin	Generations examined[a]	Copy numbers of repeats
Ky-1	Japanese new MB case	3, 5, 7, 8	9, 9, 9, 9
Ky-2	Japanese relapse MB case	4, 5, 6	11, 11, 11
Th-53	Thailander new MB case	3, 7, 11	14, 14, 14
Ze-2	Japanese relapse MB case	1, 3	11, 11
Ho-4	Japanese relapse MB case	Biopsy, 1, 2, 4	10, 10, 10, 10
Ko3-2	Korean new MB case	Biopsy, 1, 2, 4	13, 13, 13, 13
Ku-3	Japanese relapse MB case	Biopsy, 1, 2	10, 10, 10
Ku-6	Japanese relapse MB case	Biopsy, 1, 3	16, 16, 16
Ts-1	Japanese new MB case	Biopsy, 1, 2	9, 9, 9
Ze-4	Japanese relapse MB case	Biopsy, 1, 2, 3, 4	10, 10, 10, 10, 10
Ze-5	Japanese relapse MB case	Biopsy, 1, 2, 3	11, 11, 11, 11, 11

[a] Biopsy, bacilli from clinical material; generation 1 to 11, passage time in nude mouse footpad.

Samples from patients. To clarify whether the TTC genotype in one patient varies or not, genotypes of the bacilli obtained from various lesions of one patient were compared. Slit-skin smear samples from 49 lesions of 22 patients in North Maluku and North Sulawesi were obtained. Those patients in North Sulawesi included many refugees from North Maluku. Samples were collected in the same manner as is used for routine slit-skin smear testing for bacterial index examination. The sample on the disposable surgical blade was soaked in 70% ethanol and kept at room temperature until use. The sample was removed from the blade and collected as a pellet by centrifugation at 10,000 × *g* for 20 min in 70% ethanol and then washed with phosphate-buffered saline. The template was prepared by treatment with lysis buffer as mentioned above, and then the TTC genotype was examined.

Samples from household patients. TTC genotypes of the bacilli from patients living in the same dwelling were examined. Samples of five groups were collected as follows. Cases 1 and 2 (a pair of patients with household MB cases) consisted of one son and his father. Case 3 was a household case consisting of one son treated for 2 months, a new case of infection of his father, and three new cases of infection of his sister and brothers. Case 4 (a household case) consisted of one son treated for 4 months and one new case of infection of his brother. Case 5 consisted of one patient treated for 9 months and two new cases of infection of his brothers (see Table 4). Samples were collected from at least two lesions of each patient. The genotype of each isolate was examined as described below.

Samples from *M. leprae* inoculated in nude mice. To clarify the stability of the locus, we used various resources, including clinical material and different generations of serial passages in nude mice (13, 14), for comparisons of the numbers of TTC repeats within 11 strains of *M. leprae*. Template DNAs of each sample were prepared from the bacillary suspensions (which were preserved at −84°C). Each strain was maintained in nude mice footpads, with serial passages every 12 months (Table 1).

Preparation of template DNA and sequencing analysis. Templates from nasal swab materials, slit-skin samples, and bacillary suspensions were prepared by treatment with lysis buffer at 60°C overnight as described previously (5), and TTC repeat regions were amplified by PCR with the primers indicated by Shin et al. (17). Copy numbers of TTC repeats were examined by the direct sequencing of the PCR products. Briefly, the regions flanking TTC repeats were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis.). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). The forward primer was used in all sequencing reactions, since (according to the results of our preliminary study) the nucleotide sequences of interest detected by the reverse primer were deduced to be identical with those detected by the forward primer (data not shown).

Ethical approval. Informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee of National Institute of Infectious Diseases, Tokyo, Japan. Bacillary samples of nasal mucus and slit-skin smears were collected when informed consent was obtained.

RESULTS

Stability of TTC copies. Laboratory-maintained isolates showed various TTC genotypes. The copy numbers of TTC repeats in each isolate from the nude mice showed no changes and remained stable after serial passages in nude mice footpads; they also remained unchanged between the isolates from bacilli from clinical material and the corresponding isolates obtained from nude mice footpads (Table 1). Th-53 isolate was transferred with nine passages in nude mice footpads, but the number of the repeats did not change at all.

Genotype of the bacilli on the nasal mucus. The results of a previous study by Saeki et al. indicated that 28.2% (92/326) of Gamtala's villagers and 25.2% (68/270) of Lolori's villagers carried leprosy bacilli on the surface of their nasal cavities (16). *M. leprae*-positive PCR products for TTC genotyping were obtained from 48 samples out of 92 previous *M. leprae*-positive samples from Gamtala and 49 samples out of 68 previous *M. leprae*-positive Lolori's samples. Of 105 dwellings, there were 8 houses in Gamtala and 12 houses in Lolori in which more than two PCR-positive individuals carried the bacilli on the surface of their nasal cavities (Table 2). Residents in five houses in Gamtala showed different TTC genotypes of 10, 11, 12, and 13 copies. Residents in 11 houses in Lolori harbored different TTC genotypes from each other; their TTC genotypes were 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, and 24 repeats. The TTC repeats of the bacilli from the new MB case in Gamtala consisted of 10 copies, but the bacilli from his family contacts showed 11 and 13 copies of TTC repeats. The bacilli from a patient who had had contact with a PB case in house number 68 of Lolori showed 12 copies, but the genotype of the strain from this PB patient showed 14 copies (Table 2). In this area, the cases with 10 copies of TTC repeats were predominant; cases with 13 copies were the second populous group (Table 3).

Genotype of the bacilli in the lesions. For all patients examined, samples from different lesions of each patient showed identical genotypes. The TTC genotype with 10 copies was also dominant in the patients from North Maluku and North Sulawesi. The smallest number of repeats was 8; the largest was 29. In addition, other genotypes (such as 9, 11, 12, 13, 14, 15, 16, and 18 TTC copies) were detected. The frequency of each TTC genotype observed in samples from the nasal cavities of the residents and lesions of the patients is shown in Table 3.

TABLE 2. TTC genotypes of *M. leprae* detected from the surfaces of nasal mucosa of residents living in the same house

Village	House	TTC genotypes
Gamtala	9	10,[a] 11, 13, 13
Gamtala	15	13, 13
Gamtala	25	13, 13
Gamtala	78	10, 10
Gamtala	82	10, 13[b]
Gamtala	97	10,[c] 10, 13, 13, 13
Gamtala	99	10,[b] 10,[b] 10, 10, 13
Gamtala	100	10,[c] 10,[c] 10, 10, 12
Lolori	2	10, 18
Lolori	4	18, 18
Lolori	7	10, 12,[b] 18
Lolori	19	9, 12
Lolori	21	10, 14
Lolori	41	10, 13, 13
Lolori	49	8, 13
Lolori	54	13, 16
Lolori	62	12, 13
Lolori	66	11, 12
Lolori	68	12, 14[d]
Lolori	71	10, 24

[a] Newly detected MB case.
[b] Nerve enlargement without skin lesion.
[c] MB case (released from registration).
[d] PB case (registered).

Comparison of TTC genotypes among patients in a dwelling. The TTC genotypes of *M. leprae* in household leprosy cases were compared. The genotypes of one pair (the son and his father; case 2) indicated clearly independent genotypes from each other (10 copies in the son and 18 copies in the father). Bacilli of one MB case (the patient treated for 9 months; case 5) showed 12 copies of TTC repeats, while his brother harbored bacilli with 14 TTC repeats and the bacilli from another brother showed 12 TTC repeats. Another three groups of household cases (cases 1, 3, and 4) showed identical TTC genotypes within the family (Table 4).

TABLE 3. Frequency of each genotype observed in residents and patients

No. of repeats	Genotype frequency		
	Nasal mucus	Patient lesion[a]	Total
7	3		3
8	4	2	6
9	3	2	5
10	35	15	50
11	6	1	7
12	7	4	11
13	26	5	31
14	6	2	8
15		2	2
16	1	1	2
17	1		1
18	3	1	4
24	2		2
29		1	1
Total	97	36	133

[a] Includes 14 patients of household cases listed in Table 4.

TABLE 4. TTC genotypes of *M. leprae* obtained from household leprosy cases[a]

Case	Patient (TTC genotype) in supposed index case	Patient(s) (TTC genotype) in other household cases
1	Father (10)	Son (10)
2	Father (18)	Son (10)
3	Son (13)	Father (13), daughter (13), son (13) Son (13)
4	Son (8)	Son (8)
5	Son (12)	Son (12), son (14)

[a] At least two samples from different lesions were examined from each patient.

DISCUSSION

Elucidation and understanding of the source and the routes of transmission of *M. leprae* are essential in developing measures to prevent an infection. Previous seroepidemiological studies indicated widespread *M. leprae* infections within a population (1, 2, 10, 18), and studies by PCR on the distribution of the bacilli also found that many individuals in areas in which leprosy is endemic carried *M. leprae* on the surface of their nasal cavities (11, 16, 18). These studies suggested the presence of an infectious source other than that of a patient within the same dwelling. The aim of this study was to clarify microbiologically whether or not MB cases in the same dwelling represent the main source of infection.

Establishing a methodology to discriminate the isolates of *M. leprae* is fundamental for these purposes. Although many attempts have been made to subtype *M. leprae* isolates by genomic divergence (4, 9, 19), no useful methods for epidemiological analysis have been developed. Recently two genomic polymorphisms successfully discriminated isolates of *M. leprae* (12, 17). One of the authors (M. Matuoka) discovered that *M. leprae* isolates could be divided into two subtypes on the basis of the polymorphism in the *rpoT* gene. The geographical distribution of each genotype in the world was biased and seemed to be related to prehistoric movement of the human race (12). Nevertheless, the genomic diversity of the *rpoT* cannot be used for epidemiological tracing of the transmission of leprosy bacilli. Genotyping to compare diversity of short-tandem-repeat loci on the basis of PCR is feasible for molecular epidemiological analysis, since *M. leprae* is not cultivable and shows very low levels of diversion in genomic DNA (6). Variety in the copy numbers of TTC repeats can be used to classify *M. leprae* into a considerable number of subtypes and discriminate isolates for each leprosy case.

For subtyping the organisms, genetic polymorphisms must remain stable during a few rounds of infection. All isolates transferred in 1 to 11 passages in nude mice retained the same TTC genotype. Because of the wide variation and stability of the TTC repeats, genotyping of the bacilli by the TTC polymorphism proved to be satisfactory for epidemiological analysis of leprosy and discrimination of *M. leprae* distributed in areas of endemicity.

It is reasonable to assume that if the index case in the same dwelling is the source of infection, the genotypes detected in the house should be identical among the household members. In this study, various types of TTC genotypes were detected from nasal mucosa of the villagers in areas of endemicity.

However, our results clearly demonstrated that there were families with different TTC genotypes of *M. leprae* on the surface of nasal cavities among the residents in the same dwelling. Therefore, the results of the investigation suggest that these residents are contaminated by bacilli with different genotypes. No variations in genotype among the isolates obtained from various lesions in the same patient were shown. This result consequently enables comparisons of the genotypes of bacilli obtained from different patients. We had identified the existence of TTC genotypes of *M. leprae* that differed between the newly detected family contacts and the supposed index case patient. These results strongly suggest that the bacilli did not originate from a single patient in the dwelling and also indicate the exposure of the family members to infectious sources out of the dwelling. Previous seroepidemiological studies suggested that for the majority of cases, the possible source of infection might be in the environment rather than in direct contact with leprosy patients (1, 2, 10). The findings by PCR, which revealed the wide distribution of the bacilli among the residents in areas of endemicity, also indicated that the transmission of the bacilli was not only from the leprosy patients (11, 16, 18). The present study strongly supports these assumptions respecting the infectious source(s). Although many epidemiological observations indicated that the household contact was the risk factor for the development of leprosy (7, 15), on the other hand, many new cases among people without any known household contacts with patients were detected (7). It is therefore not necessarily the case that the household patient is the only source of infection of leprosy bacilli. The tendency seen of the accumulation of patients in some families might be attributed to other conditions such as susceptibility to leprosy infection, which is related to genetic predisposition as well as to acquired factors (3).

Two groups of the household leprosy cases showed apparently different TTC genotypes between a father and his son and among brothers. The inconsistency of the genotypes between *M. leprae* isolates obtained from household cases of patients living in the same dwelling clearly indicates that these patients are not always the source in infections of the other family members. Though the members of the other groups of leprosy cases showed the same genotype, whether those people were truly infected by the patient in the house was unclear. The presence of the same genotype in two cases doesn't necessarily imply the infection was transmitted from a patient to family contacts, for some TTC genotypes, such as those of 10 and 13 repeats, were widely distributed in the areas. Other polymorphisms which can discriminate within a given TTC genotype are needed to elucidate this problem. Better epidemiological analysis could be done by the combination of various genotyping techniques. However, TTC genotyping enabled the subtyping of *M. leprae* into more types than *rpoT* genotyping. It is expected that other short-polymorphic-tandem-repeat loci exist in *M. leprae* genome, in similarity to those observed in investigations of *M. tuberculosis* (8). A combination with genotyping using other polymorphisms might be a useful tool for precise epidemiological analysis. Other genotyping measures depending on other short-polymorphic-tandem-repeat loci are required. The frequency of 24 or 25 TTC repeats was the highest in the previous study, which examined *M. leprae* isolates obtained in Cebu, Philippines (17). Bacilli with 10 copies of TTC repeats were most frequently isolated in the present study, and the bacilli with large numbers (such as 37) of TTC repeats were not detected (Table 3). It is of interest to compare the frequencies of each genotype in different areas, since the results of a previous study indicated that the spread of the bacilli with specific genotypes was consistent with migration of some human groups (12).

The evidence resulting from the present molecular epidemiological study indicated the existence of an infectious source other than patients in the same dwelling. Wide distribution of the bacilli among residents (11, 16, 18) and a high positive ratio of anti-PGL-1 antibody among healthy residents (2, 10) suggested that the bacilli existed in certain sources to which people were commonly exposed. Taking these results into consideration, the environment seems to be the most likely infectious source. However, exactly what the infectious source is has not been elucidated so far.

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IL-10 Treatment of Macrophages Bolsters Intracellular Survival of *Mycobacterium leprae*[1, 2]

Yasuo Fukutomi, Masanori Matsuoka, Fumishige Minagawa, Satoshi Toratani, Gregory McCormick, and James Krahenbuhl[3]

ABSTRACT

In these studies, metabolically active *Mycobacterium leprae* were maintained for as long as 8 weeks in monolayer cultures of mouse peritoneal macrophages (MΦ). Supplemental IL-10, but not TGF-β, bolstered, directly or indirectly, *M. leprae* metabolism in mouse MΦ. In the cell culture system temperature setting is extremely important and 31 to 33°C incubation temperature was more permissive than 37°C. Acid fast staining and transmission electron microscopy (TEM) of intracellular *M. leprae* revealed visible elongation of bacilli cultured under the above ideal conditions.

RÉSUMÉ

Mycobacterium leprae n'a jamais encore été vraiment cultivé sur milieu artificiel. Comme *M. leprae* préfère vivre *in vivo* à l'intérieur de la cellule, nous avons exploré la croissance *in vitro* de *M. leprae* dans des cultures de phagocytes mononucléés ou de macrophages, qui représentent la cellule hôte favorite du bacille de la lèpre. Notre approche expérimentale a tenu compte des faiblesses de ce type d'expérimentation : à savoir la viabilité de l'*inoculum* et la longue durée de multiplication de *M. leprae*. Le but n'était pas ici de démontrer une augmentation mesurable et significative du nombre de *M. leprae*, mais plutôt le maintien prolongé du métabolisme de *M. leprae*, en mesurant l'oxydation de l'acide palmitique radiomarqué, comme indicateur de viabilité.

Des *M. leprae* ayant un métabolisme actif ont été maintenues jusqu'à 8 semaines dans des cultures mono-couches de macrophages péritonéaux de souris. L'ajout d'IL-10, mais pas de TGF, a augmenté, directement ou indirectement, le métabolisme de *M. leprae* dans les macrophages de souris. Le réglage de la température des cultures cellulaires est extrêmement important et une température d'incubation de 31–33°C était plus favorable qu'une température de 37°C. La coloration acido-alcoolo-résistante et la microscopie électronique à transmission des *M. leprae* a permis de mettre en évidence des élongations bien visibles parmi les bacilles cultivés dans les conditions idéales mentionnées ci-dessus.

RESUMEN

Mycobacterium leprae todavía no se ha podido cultivar en medios artificiales. Como esta bacteria prefiere una existencia intracelular *in vivo*, en este estudio exploramos el crecimiento *in vitro* de *M. leprae* en cultivos de fagocitos o macrófagos, el huésped preferido del bacilo de la lepra. Reconocemos que nuestro diseño experimental conlleva dos problemas: la viabilidad de *M. leprae* en el inóculo, usualmente baja, y el prolongado tiempo de división de la bacteria. Aunque no esperábamos encontrar un incremento sustancial en los números de *M. leprae*, sí pensamos poder observar cambios en su metabolismo midiendo la oxidación del ácido palmítico radiactivo como un marcador de viabilidad. Encontramos que la bacteria se mantuvo metabolicamente activa hasta por 8 semanas en los cultivos de los macrófagos peritoneales de ratón. La adición de IL-10 pero no de TGF, apoyó, directa- o indirectamente el metabolismo de *M. leprae* en los macrófagos de ratón. Las condiciones de incubación de los cultivos fueron muy importantes y la temperatura de 31–33°C fue más permisiva que la temperatura de 37°C. La tinción para ácido-resistentes y la microscopía electrónica de transmisión revelaron cierto grado de alargamiento de los bacilos bajo las condiciones óptimas de cultivo de los macrófagos.

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[3] Y. Fukutomi; M. Matsuoka; F. Minagawa; S. Toratani, Leprosy Research Center, National institute of Infectious Diseases, Tokyo, Japan; G. McCormick; and J. Krahenbuhl, The Gillis W. Long Hansen's Disease Center Laboratory Research Branch, Louisiana State University, Baton Rouge, Louisiana, U.S.A.
Reprint requests to: Dr. Y. Fukutomi, fukutomi@nih.go.jp

In the 130 years since the discovery of *Mycobacterium leprae* as the causative agent of leprosy, a large number of attempts have been made to cultivate this obligate intracellular pathogen in cell-free media ([6, 30]). None of these efforts have fulfilled the criteria for success suggested by John Hanks ([18]), especially the confirmation of findings by a second laboratory. The inability to culture the leprosy bacillus has undoubtedly hindered almost every aspect of leprosy research, and thus, our understanding of this disease lags behind that of many others of bacterial etiology.

Reasoning that the intracellular milieu would best suit the multiplication of the obligate intracellular leprosy bacillus, as an alternative to culture in axenic medium, a number of attempts have been made to cultivate *M. leprae* in various types of cultured cells. Fieldsteel and McIntosh ([10]) employed a range of rat, mouse, and human tissue but found no evidence of multiplication. The preferred host cell for the leprosy bacillus, however, appears to be the mononuclear phagocyte or macrophage (MΦ) and a number of unsuccessful attempts have been made to grow *M. leprae* in MΦ ([5, 8, 16, 24, 27, 31, 34, 36, 41]). The present approach also employs MΦ but is novel in that we employed conditions that inhibit innate antimicrobial functions in infected mouse MΦs to bolster the intracellular survival of *M. leprae*. Moreover, we had a number of advantages over the previous attempts by others including: unique resources, previously unavailable to other workers in the form of fresh, highly viable *M. leprae* ([42]), sensitive techniques for measuring and comparing the metabolic activity of *M. leprae* ([13]) and the extensive experience of our two laboratories in studying the relationship between the MΦ and the leprosy bacillus ([2, 14, 15, 39, 40]).

MATERIALS AND METHODS

Maintenance of a viable *M. leprae* inoculum. The Thai-53 strain of *M. leprae* ([26]) was maintained in continuous passage in athymic nu/nu mice (Crea Co., Tokyo, Japan) by inoculation of 1×10^7 freshly harvested bacilli into both hind footpads. At approximately nine months post, footpads were processed to recover *M. leprae* by Nakamura's method with a slight modification ([28]). Briefly, tissue was minced and homogenized with Hanks' balanced salt solution (HBSS) containing 0.05% Tween 80. The homogenate was centrifuged at $150 \times g$ for 10 min and supernatant of the sample homogenate was treated with 0.05% trypsin at 37°C for 60 min. The suspension was centrifuged at $4000 \times g$ for 20 min and sediment was re-suspended in HBSS followed by treatment with 1% sodium hydroxide at 37°C for 15 min. The treated material was washed and re-suspended in HBSS at the desired bacillary concentration. Bacillary number in each footpad was enumerated individually according to standard techniques ([37]).

Cytokines. Murine recombinant IL-10 was obtained from Genzyme Corp. T cell growth factor β (TGF-β) was obtained from Kurashiki Bouseki, Kurashiki, Japan). Both cytokines were stored at –80°C until use.

Mouse MΦ culture. Mouse peritoneal resident cells (approximately 50% MΦ) were harvested from retired ICR or Swiss White (SW) mice and suspended as previously described ([2]) at a concentration of 2×10^6/ml in RPMI 1640 (GIBCO, Grand Island, NY) + 15% fetal bovine serum (HyClone Laboratories, Logan, UT) + 25 mM hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (GIBCO), 0.2% NaHCO3 (GIBCO), 2 mM glutamine (Irvine Scientific, Santa Ana, CA), and 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). 0.5 ml was seeded into 24 well tissue culture plates (Corning) containing 16 mm LUX coverslips (Miles Laboratory, Napierville, IL). After overnight adherence of the cells, MΦ monolayers were obtained after washing non-adherant cells from the coverslip with Hanks Balanced Salt Solution (HBSS) leaving approximately 1×10^6 MΦ adhered per coverslip.

Infection of MΦ with *M. leprae*. Purified mouse MΦ monolayers were infected with fresh *M. leprae* suspended in 0.5 ml medium at a multiplicity of infection of 20:1. After 4 hr incubation, non-phagocytized bacteria were removed by washing and the cultures reincubated in 1.0 ml media supplemented with the appropriate cytokine in 5% CO_2 at the appropriate experimental temperatures ([2]). Media was changed and, where appropriate, cytokines replenished at 5 day intervals.

Radiorespirometry (RR). The MΦ were lysed with 0.1 N NaOH to release the *M.*

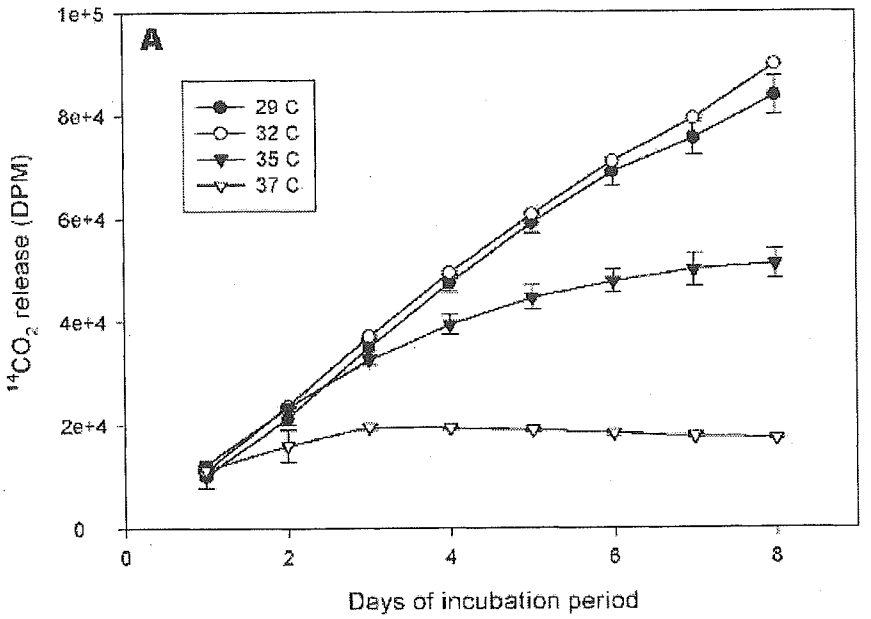

FIG. 1A. Effect of incubation temperature on metabolic activity (RR) of *M. leprae* in axenic media. Each vial was inoculated in triplicate with 2×10^7 freshly harvested, nude mouse-derived *M. leprae* and incubated at different temperatures. In the experiment shown, metabolic activity, shown as $^{14}CO_2$ release, was monitored every day for 8 days. These findings are representative of dozens of related experiments showing the detrimental effects of 37°C for *M. leprae*.

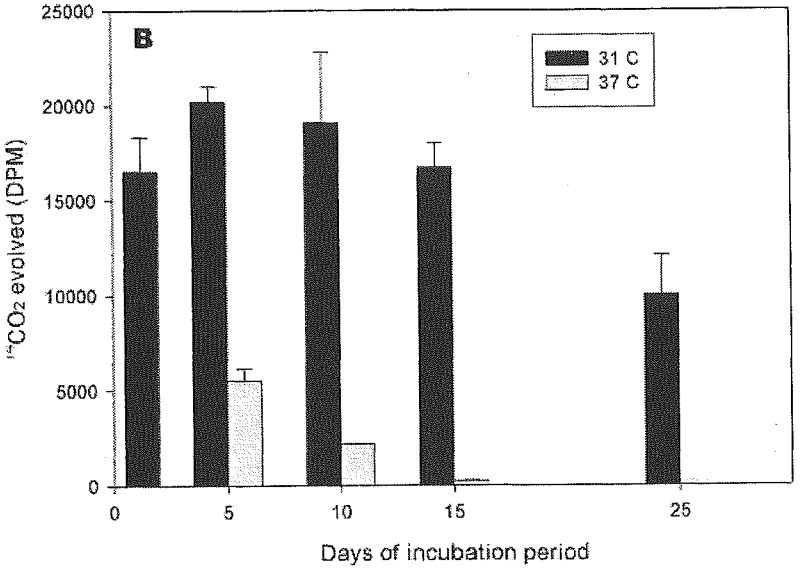

FIG. 1B. Metabolic activity of *M. leprae* in MΦ cultured at 31°C or 37°C. *M. leprae* were released from infected MΦ on the days shown (in quadruplicate) and inoculated into RR vials. The data shown represent RR data obtained after 7 days.

leprae, and the viability of the bacilli was determined by evaluating the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ by radiorespirometry as described previously ([13]). Total isotope release was usually analyzed after one week of incubation at 31°C ([2]).

Staining of *M. leprae*-infected MΦ. Coverslips of *M. leprae*-infected adherent MΦs were prefixed with absolute methanol, and acid-fast stained Photomicrographs were taken using a Nikon Optiphot microscope using an oil immersion Plan APO 100 lens.

Transmission electron microscopy (TEM). MΦ monolayers on coverslips were pre-fixed in 2% glutaraldehyde/0.1 M Na-cacodylate buffer followed by post-fixation with osmium tetroxide/K-CN and endoblock staining with uranyl acetate. The

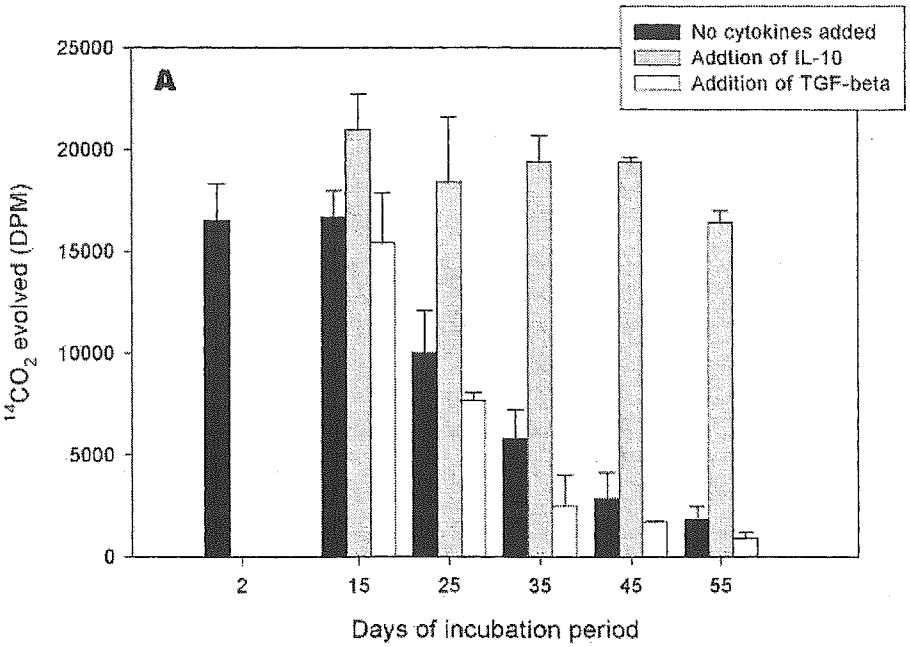

FIG. 2A. Metabolic activity of *M. leprae* in MΦ cultured in the presence of IL-10 or TGF-β. *M. leprae* infected MΦ were incubated at 31°C in the presence or absence of 2 U/ml IL-10 or 10 ng/ml TGF-β and bacilli were released from infected MΦ on the days shown (in triplicate) and inoculated into RR vials. The data shown represent RR data obtained after 7 days.

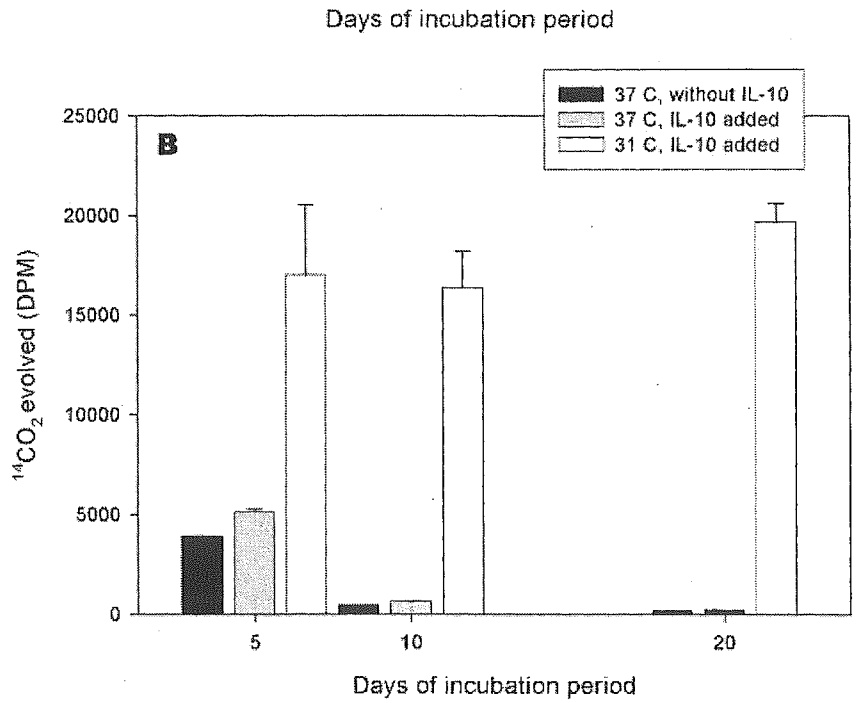

FIG. 2B. Comparison of metabolic activity of *M. leprae* in MΦ cultured at 31°C and 37°C in the presence of IL-10. *M. leprae* infected MΦ were incubated at 31°C or 37°C in the presence or absence of 2 U/ml IL-10 and bacilli were released from infected MΦ on the days shown (in triplicate) and inoculated into RR vials. The data shown represent RR data obtained after 7 days.

specimen was then dehydrated with ethanol, embedded in Epon-Araldite, sectioned at 90 nm, stained with uranyl acetate-lead citrate and viewed with a Philips 410 TEM as previously described ([39]).

RESULTS

***In vitro* temperature preferences of *M. leprae*.** *M. leprae* clearly prefers cooler incubation temperatures. As shown in Figure

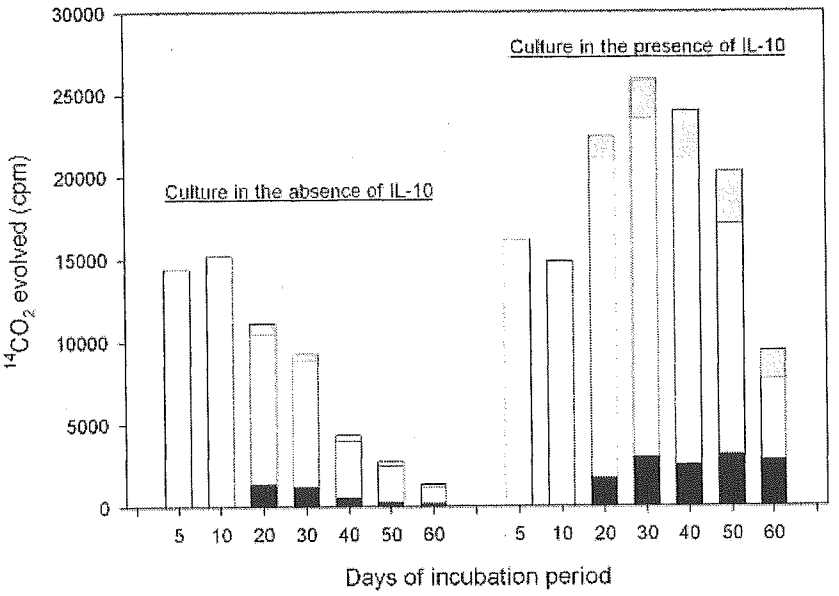

FIG. 3. Metabolic activity of *M. leprae* in MΦ cultured in the presence of IL-10. *M. leprae* infected MΦ were incubated at 31°C in the presence or absence of 2 U/ml IL-10. On the days shown (starting on day 20) we accounted for the metabolic activity of all bacilli in each well (free bacilli or detached infected cells in supernatant plus bacilli present in attached MΦ). The middle open portion of each bar represents RR activity of bacilli released from adherent MΦ. The upper shaded portion of each stacked bar represents RR activity of bacilli found in the supernatant when the media was changed 5 days previously (day 15, 25, 35, etc.). The lower black portion of each bar represent RR of bacilli in the supernatant on the day of the harvest.

1A, in axenic culture, it was apparent that 37°C was not an ideal temperature to demonstrate sustained viability. Incubation at 35°C was more supportive than 37°C, and results at 29°C and 32°C were indistinguishable but even more ideal.

Similarly, intracellular *M. leprae* thrives better at cooler temperatures. Mouse MΦ appeared to function normally at 33°C and even 31°C, as judged by attachment to plastic and phagocytic capacity, although they did not spread as well at these lower temperatures as they do at 37°C. In the experiment depicted in Figure 1B, infected MΦs were incubated at either 31°C or 37°C and at 5 day intervals released bacilli were studied by RR for an additional 7 days. The detrimental effects of incubation at 37°C on *M. leprae* metabolism were apparent by day 5. In marked contrast, *M. leprae* cultured in MΦ at 31°C thrived for at least 15 days and retained most of its viability after 25 days in MΦ maintained at the lower temperature.

Effects of cytokines on viability of *M. leprae* in MΦ. Supplementation of the infected MΦ culture medium with 2 U/ml murine IL-10 was clearly associated with sustained viability of intracellular *M. leprae*. In the more prolonged experiments depicted in Figure 2A and 2B, *M. leprae* steadily lost viability in control MΦ at 31°C and 37°C. In contrast, in MΦ incubated in the presence of IL-10, *M. leprae* maintained their viability, but only at the permissive temperature of 31°C. (Figure 2A and 2B). As shown in Figure 2A, addition of TGF-β to the infected MΦ had no effect on the viability of *M. leprae*.

Experiments were run to account for all of the *M. leprae* in the long term cultures, assuming that during prolonged culture some infected MΦ may detach or lyse, releasing their bacilli. In the experiment depicted in Fig. 3, media was changed as usual every 5 days and data points recorded every 10 days. In order to account for bacilli released from MΦ or bacilli in "detached" MΦ we collected and saved the "old" media at 4°C at the time it was changed (midpoints of the 10 day time points plotted at 20, 30, 40 days, etc.). The viability of bacilli in the individual MΦ monolayers and in the MΦ detached from the monolayers are shown separately and as a total. These data show that only a few *M. leprae* were released or infected cells detached into the supernatant media, and the cumulative radio-respirometry (RR) results from individual wells confirmed the ability of IL-10 treatment to sustain intracellular viability of *M. leprae*.

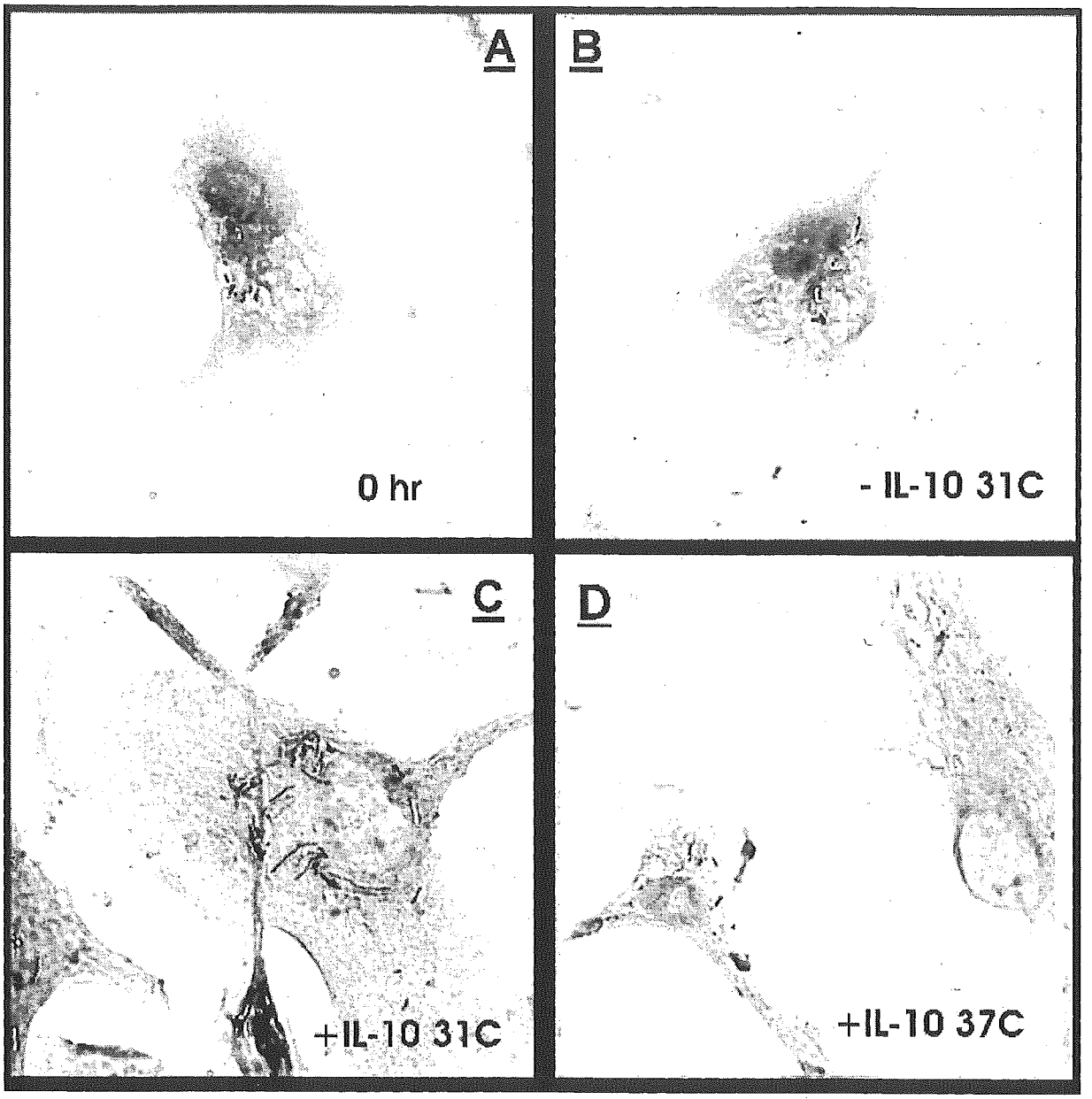

FIG. 4. Elongation of *M. leprae* in mouse MΦ cultured in the presence of IL-10 at 31°C or 37°C. The cells were acid fast stained and observed under light microscopy with magnification at ×1000. Panel A = 0 hr (31°C). B = 4 wks at 31°C without IL-10. C = 4 wks at 31°C + IL-10. D = 4 wks at 37°C + IL-10. Figures are representative of observations from 2 experiments.

Morphological evaluation of *M. leprae* with sustained metabolic activity in MΦ. The morphological characteristics of *M. leprae* maintained in prolonged culture in mouse peritoneal MΦ were observed with light and electron microscopy. Elongated *M. leprae* were only observed under conditions where infected MΦ were maintained at 31°C in the presence of IL-10. As shown in Fig. 4, acid fast staining of infected MΦ at 4 weeks revealed that at 31°C in the presence of IL-10, many of the intracellular *M. leprae* were clearly elongated in comparison to those seen at 0 time or in MΦ maintained at 31°C without IL-10 (Figure 4A, B, C). At 37°C elongation of bacilli was not observed regardless of the presence of IL-10 (Fig. 4D). Under the transmission electron microscope (TEM), elongation was even more apparent. Not all bacilli in the 31°C, IL-10 group were observed to be elongated, as this required all bacilli to be sectioned through their long axis; but examination of dozens of infected cells in 2 experiments revealed elongated cells (8 to 10 μ) only in the 31°C, IL-10 group. *M. leprae*

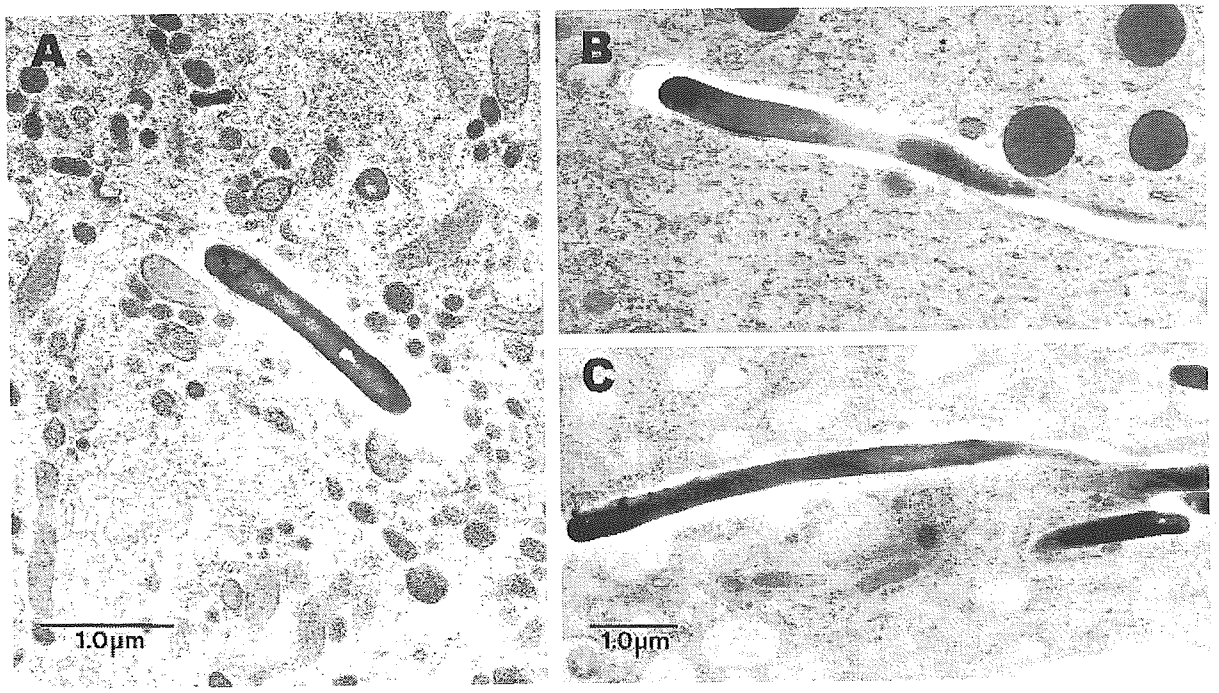

FIG. 5. Elongation of *M. leprae* in macrophages cultured *in vitro*. Mouse macrophages were infected with *M. leprae* followed by washing and incubation in the presence or absence of IL-10 at 31°C for 4 weeks. The cells were fixed, cut and observed under transmission electron microscopy. A, control (without IL-10); B, C, +IL-10. Bars in each panel = 2 μm.

in the control group were consistently 2 to 4μ in length (Fig. 5).

DISCUSSION

Our goals in this study were limited. Convincing evidence of actual intracellular multiplication of *M. leprae* would require at least a 10-fold increase in bacillary numbers. With a calculated multiplication cycle of 12.8 days in the mouse footpad model ([37]), this minimally acceptable increase in numbers would be difficult to demonstrate in a few weeks of MΦ tissue culture. However, the present study did show that the metabolism, and presumably the viability ([42]), of *M. leprae* could be sustained under culture conditions which also appeared to support the intracellular elongatiion of the leprosy bacillus.

In vivo M. leprae is able to enter and survive in a wide variety of tissues and cell types ([24]). Attempts to culture *M. leprae* in tissue culture have included the use of numerous cell lines derived from humans, rats, and mouse tissue with no evidence of multiplication ([10, 27]). The MΦ, the preferred host cell for the leprosy bacillus, offers an advantage over tissue culture cell lines since MΦ actively phagocytize *M. leprae* and, unlike cell lines, MΦ in culture are non-dividing adherent cells. Consequently the intracellular status of *M. leprae* over time is not confounded by an increase in host cell numbers. Chang and Neikirk ([5]) demonstrated the long term infection of mouse MΦ cultures with *M. leprae*, and a report of success in culturing *M. leprae* in MΦ was made by Garbutt ([16]), but was not confirmed by McRae and Shepard ([27]). Others reported limited, questionable, and unconfirmed success at detecting multiplication of the organism in MΦ cultures ([8, 31, 34, 41]). An exhaustive but unsuccessful attempt to cultivate *M. leprae* in tissue culture was made by Sharp and Banerjee ([36]) who employed MΦ from conventional mice and rats, nu/nu mice and rats and armadillos, rather than dividing cells and cell lines. Their *M. leprae* inocula was derived from 3 sources (human leproma, nu/nu mouse footpad and frozen infected armadillo tissue). Incubation temperature was varied from 31°C to 35°C and infected cells were maintained for up to 200 days. They rigorously evaluated any increase in leprosy bacilli and concluded that no significant multiplication occurred.

Our studies provide groundwork for fu-