津聖志：結核菌蛋白 Ag85B 由来 Peptide-25 特異的 T 細胞クローンの TCR を認識する抗イディオタイプ抗体、KN7 を用いた Th1 応答の解析、第３４回日本免疫学会、12, 2004, 札幌

H. 知的財産権の出願・登録状況

1. 特許取得　　なし
2. 実用新案登録 なし
3. その他　　なし

研究成果の刊行に関する一覧表

別紙5

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
牧野正彦	結核・ハンセン病	倉田毅編	ネオエスカ　感染症・アレルギーと生体防御	同文書院出版		2005	印刷中

別紙5

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Studies of Lipoproteins of *Mycobacterium leprae*

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The deciphering of the genomic sequence of *Mycobacterium leprae* has made possible to predict the possible lipoproteins. The consensus sequence at the N-terminal region of the protein, including the cysteine residue to which the lipid moiety gets attached, provides a clue to the search. As such, more than 20 putative lipoproteins have been identified from *Mycobacterium leprae* genomic sequence. Lipoprotein LpK (*Accession no. ML0603*) which encodes for 371 amino acid precursor protein, was identified. Expression of the protein, in *Escherichia coli* revealed a 33 kD protein, and metabolic labeling experiments proved that the protein was lipidated. The purified lipoprotein was found to induce production of IL-12 in human peripheral blood monocytes which may imply that *M. leprae* LpK is involved in protective immunity against leprosy. Pursuit of such lipoproteins may reveal insights into the pathogenesis of the disease.

Introduction

According to World Heath Organization (WHO) epidemiological survey report, the number of leprosy patients in the world was around 534000 at the beginning of 2003, as reported by 110 countries. About 620000 new cases were detected during 2002 (http://www.who.int/lep/). Inspite of the intensive leprosy control measures taken, there is no evidence as yet of a reduction in the number of new cases [1)]. The situation implies that there is a need to develop new vaccines and immunotherapeutic tools to control the disease. Moreover there is increased concern about the disease due to the complications due to severe reactions, peripheral nerve injury due to the tropism of the bacilli to invade Schwann cells [2-4)] and emergence of drug resistant bacilli [5)].

Bacterial lipoproteins containing N-acyl diglyceride-cysteine residues, flanked by characteristic amino acids motif that are required for post-translational processing via the signal peptidase II [6, 7)], have been extensively studied in Gram-positive and Gram-negative bacteria. Membrane located 17 kD lipoprotein of *Francisella tularensis* reported by Sjosted *et al.* was found to be T cell stimulatory [8, 9)]. Lipoproteins released by pathogenic *E. coli*, *Salmonella typhimurium* and *Yersinia enterocolitica*, were found to induce proinflammatory cytokine in macrophages and ameliorate pathologic changes associated with gram negative bacterial infection in mice [10)].

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Borrelia burgdorferi and *Treponema pallidum*, the etiological agents of lyme disease and syphilis, respectively, are known to possess abundant lipoproteins [11], which act as major antagonists with the ability to influence both innate and adaptive immune responses during infection [12]. The only two well studied mycobacterial lipoproteins, are the 19 kD and 38 kD lipoproteins of *Mycobacterium tuberculosis* [13-15]. These lipoproteins are therefore presumed to be involved in the host responses, inducing interleukin-12 (IL-12) from the host cells. Since IL-12 has T cell stimulatory properties, which in turn elicits production of interferon- γ (IFN- γ), and facilitates development of Th1 cells [16-18], these lipoproteins may be involved in the induction of cellular responses to mycobacteria and thereby contributing to the development of protective immunity[19, 20]. Identification of lipoproteins in *M. leprae* seems inevitable especially in terms of host defense and for the development of new vaccines against leprosy.

Analysis of a *M. leprae* lipoprotein

To date, relatively few lipoproteins of mycobacteria have been described. The database of the *M. tuberculosis* genome (http://www.sanger.ac.uk/Projects/M_tuberculosis/) revealed that there are about a hundred putative lipoprotein coding genes, but only about 40 genes have been identified in *M. leprae* genome [21] and almost half of the genes identified are pseudogenes. Table 1 shows the list of the putative lipoproteins. One of the predictable lipoprotein was found to be partially homologous to the precursor of the glutamine binding protein, the other one was a possible transport lipoprotein and the third one was a putatitve secreted protease. But all other lipoproteins had no homology to any other protein of known function. One of the more interesting candidates is the gene annotated as *lpk* (Accession No. ML0603) [22]. The N-terminal residues of LpK showed typical features of a signal peptide with a consensus sequence (MISALMVAVAC) for the lipid modification. A sequence homologue of *lpk* was identified in the *M. tuberculosis* genome database using the BLASTN search tool. *M. tuberculosis* Rv 2413c (EMBL:AL123456, 316 amino acids) has 83.5% identity in the 316 amino acid overlap. However, the homologue has no consensus sequence for lipid modification. The fact that the lipid consensus sequence was missing is quite surprising since many of the *M. leprae* genes when compared to those of *M. tuberculosis* genes are pseudogenes as analyzed from the gene databases [21]. This fact may indicate that this lipoprotein may be specific to *M. leprae* and have a significant role in bacteria, specifically related to the unique features of the organism such as proclivity for Schwann cell invasion or development of reactions. Since it is not feasible to obtain adequate amount of protein from *M. leprae* for analyses, the gene was cloned and the protein expressed and purified in *E. coli* (Fig.1). The basic lipoprotein nature of LpK was verified experimentally. Metabolic labelling of the bacterial protein with radioactive glycerol provided presumptive evidence of a covalent linkage of lipid to LpK.

Murine experiments with infectious pathogens, indicate that IL-12 plays an important role in inititation and regulation of the T cell responses such as Th1 [23, 24]. *In vitro* experiments with *M. tuberculosis* suggested that IL-12 is induced rapidly after infection [16, 25, 26], and in *in vivo* IL-12 was crucial for the development of protective immunity against tuberculosis [27]. When we examined whether IL-12 was inducible by LpK in human monocytes, LpK induced IL-12 at a significantly high level, a level that could be maintained even in the presence of polymyxin B (Fig. 2). Another *M. leprae* putative lipoprotein (gene product of Accession No. ML1699) was expressed in inclusion bodies of *E. coli*. The purified protein, of molecular weight 39 kD, did not induce any significant amount of IL-12 in human monocytes. The reason for non-inducing capability of the purified 39 kD protein, may be the lack of lipidified region, although the exact reason remains unclear.

Discussion

M. tuberculosis 19 kD lipoprotein is both cell wall associated and secreted lipoprotein which stimulate proliferation of human T cells and promotes neutrophil priming and activation [14,28]. It is also known to induce apoptosis in macrophages through TLR2 ligation [29]. Recently, the synthetic lipopeptide consisting of the N-terminal portion of *M. leprae* 19 kD lipoprotein is shown to induce apoptosis in human Schwann cells, also through TLR2 [30]. At present, TLR2 seems to be the only receptor known to be involved in signaling of bacterial lipoproteins and lipopeptides [31]. In likewise manner, TLR2 seems to be the receptor on antigen presenting cell, which is involved in *M. leprae* LpK lipoprotein signaling. But blocking of TLR2 with its antagonistic antibody does not completely inhibit the T cell activating ability of lipoprotein. Therefore other receptors as yet unknown, may be required for the signaling. Also, TLR2 seems to associate with TLR1 and recognise the native 19 kD *M. tuberculosis* lipoprotein and synthetic triacylated but not the diacylated lipopeptide [32,33]. Such type of inter-related receptors may also be worth investigating.

Display of outer surface protein A (OspA) antigen as membrane associated lipoprotein by *M. bovis* bacillus Calmette-Guerin seem to be necessary for protection against *Borrelia burgdorferi* infection (Lyme disease) [34]. But there are a few reports which considers the involvement of lipoprotein deleterious to protection against disease [35]. Therefore it would be necessary to see whether the display of *M. leprae* lipoproteins could enhance host defense-associated immunity as well as serve in protection against the disease in *in vivo*.

IL-12 production in mycobacterial diseases is known to contribute to antimycobacterial defenses [17,36,37], by triggering of interferon- γ which, in turn, can reduce, for example, the bacillary load in lepromatous leprosy patients [16]. In this respect, we can anticipate that lipoproteins may have the potential to be used as an immunotherapeutic agent against leprosy. We may have to investigate the IL-12 inducing ability of other lipoproteins of *M. leprae* and the detailed mechanism by which the signal is transduced.

In conclusion, LpK, induced the production of IL-12 which may indicate a significant role in the induction of cellular responses leading to the development of protective immunity against the intracellular organism. Although the engagement of lipoproteins in the pathogenesis of leprosy is still to be evaluated, ongoing studies are conducted to evaluate its immunogenic role on leprosy.

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TABLE 1. The putative lipoproteins of *M. leprae*[1]

No.	CDS Number *(M. leprae)*	No. of amino acid residues	Products
1	ML0136	233	Putative lipoprotein (lppX)
2	ML0246	218	Putative lipoprotein (lpqT)
3	ML0319	183	Putative lipoprotein (lpqE)
4	ML0489	556	Hypothetical lipoprotein
5	ML0557	238	Putative lipoprotein (lprG)
6	ML0603	371	Lipoprotein
7	ML0775	589	Putative lipoprotein (lpqB)
8	ML0902	239	Putative lipoprotein
9	ML1086	468	Probable transport protein
10	ML1093	285	lipoprotein
11	ML1099	202	Putative lipoprotein
12	ML1115	188	Possible lipoprotein
13	ML1116	187	Lipoprotein (lrpC)
14	ML1177	126	Possible lipoprotein
15	ML1315	194	Probable lipoprotein (lppK)
16	ML1339	525	Putative secreted protease
17	ML1427	445	Possible transport protein
18	ML1699	302	Putative lipoprotein
19	ML1966	161	Possible lipoprotein (lpqH)
20	ML2010	153	Putative lipoprotein
21	ML2446	441	Possible lipoprotein
22	ML2593	393	Putative lipoprotein (lprK)

[1]CDS from *M. leprae* Sanger database and number of amino acids in the prolipoprotein forms of the *M. leprae* lipoproteins are shown.

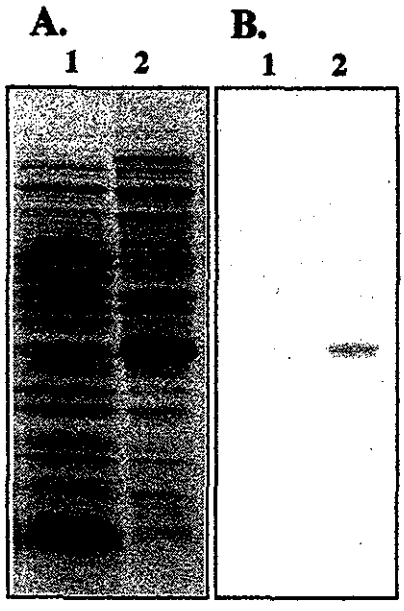

Fig. 1 : Expression and detection of *M. leprae* LpK in *E. coli*, A. Coomassie stain : 1, mock transformed and 2. *lpk* transformed *E. coli* extract. B. Western blot of the same, using monoclonal anti-His tag antibody.

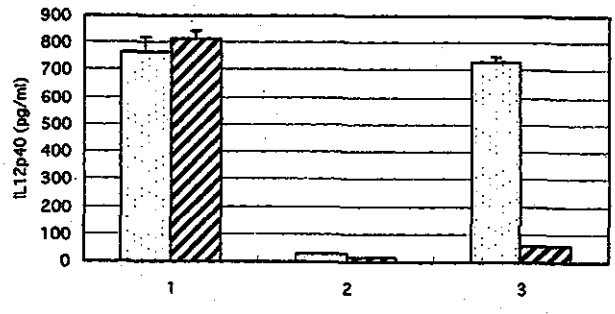

Fig. 2 : IL-12 p40 production is induced by *M. leprae* lipoprotein LpK : IL-12 p40 cytokine induction from human blood monocytes was observed using 1-LpK, 2-gene product of ML1699, 3-LPS. Hatched bar indicates the production of IL-12 p40 in the presence of polymyxin B.

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らい菌のリポ蛋白に関する研究

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ハンセン病の病原体であるらい菌の生体防御に関わる因子として、リポ蛋白に着目した。現在までに、結核菌の分子量19kDのリポ蛋白が、感染免疫反応に重要な役割をしているインターロイキン12（IL-12）を強く誘導することが報告されている。近年、らい菌のゲノムプロジェクトのデーターベースが完成され、脂質附加を受けることが予想される幾つかのリポ蛋白をコードするらい菌遺伝子を探索することができた。その結果、らい菌の33kDリポ蛋白はIL-12を強く誘導し、生体防御反応に密接に関与しているものと想定された。

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CORRESPONDENCE[1]

This department is for the publication of informal communications that are of interest because they are informative and stimulating, and for the discussion of controversial matters. The mandate of this JOURNAL is to disseminate information relating to leprosy in particular and also other mycobacterial diseases. Dissident comment or interpretation on published research is of course valid, but personality attacks on individuals would seem unnecessary. Political comments, valid or not, also are unwelcome. They might result in interference with the distribution of the JOURNAL and thus interfere with its prime purpose.

Active Surveillance of Leprosy Contacts in Country with Low Prevalence Rate[2]

ABSTRACT

For advanced control of leprosy in Pakistan where the World Health Organization leprosy elimination goal was achieved in 1996, we conducted surveillance of *Mycobacterium leprae*-seropositive patients and their contacts and drug resistant strains of *M. leprae*.

We measured anti-PGL-I antibody level in sera from leprosy patients and their contacts for early detection of *M. leprae* infection. Out of 34 leprosy patients undergoing treatment, 4 lepromatous leprosy patients were antibody positive, and 6.8 to 23.7 percent of occupational or household contacts were seropositive. Furthermore, three cases (1.2%) had a high antibody titer. For surveillance of drug resistant strains of *M. leprae*, dapsone and rifampin were targeted. Four out of 18 polymerase chain reaction (PCR) positive samples had mutation in *folP* gene, and among 10 PCR positive samples, one had a mutation in the *rpoB* gene.

These results indicate that serological analysis of patient contacts might be useful to find out high risk individuals, and there are *M. leprae* strains resistant to chemotherapeutic agents in Pakistan.

RÉSUMÉ

Dans le cadre du contrôle avancé de la lèpre au Pakistan où le programme de l'Organisation Mondiale de la Santé a atteint son but d'élimination en 1996, nous avons mené une étude d'épidémio-surveillance des patients séropositifs contre *Mycobactérium leprae*, de leurs contacts et des souches résistantes de *M. leprae* aux médicaments.

Nous avons mesuré les niveaux d'anticorps anti-PGL-I dans le sérum de patients lépreux et des personnes en contact avec ces derniers afin d'effectuer une détection précoce de l'infection par *M. leprae*. Parmi 34 patients actuellement sous traitement, 4 patients lépromateux étaient positifs à l'examen sérologique, et 6,8 à 23,7 pour cent des personnes en contact, soit professionnel, soit domestiques, furent séropositifs. De plus, 3 cas (1,2%) présentaient un titre élevé. La résistance à la dapsone et la rifampicine furent évaluées pour la surveillance des souches résistantes de *M. leprae*. Quatre des 18 échantillons positifs par PCR présentaient des mutations du gène *folP* et, parmi 10 échantillons positifs par PCR, une avait une mutation du gène *rpoB*.

Ces résultats indiquent que l'analyse sérologique des contacts proches de patients hanséniens pourrait bien être utile pour découvrir les individus à haut risque et qu'il existe des souches de *M. leprae* résistantes aux médicaments chimiothérapeutiques au Pakistan.

RESUMEN

Se hizo un estudio en Pakistán, donde la meta de la OMS de eliminación de la lepra se logró en 1996, para evaluar la evolución de los pacientes sero-positivos a *Mycobacterium leprae* y sus contactos, y para detectar cepas de *M. leprae* resistentes a las drogas antileprosas.

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Se midió la presencia de anticuerpos anti-PGL-I en los sueros de los pacientes y sus contactos para detectar la infección temprana por *M. leprae*. De los 34 pacientes en tratamiento, 4 pacientes con lepra lepromatosa (11.7%) tuvieron anticuerpos anti-PGL-I, además de que 6.8% de los contactos ocupacionales y 23.7 % de los contactos convivientes también fueron sero-positivos. Tres casos (1.2%) tuvieron anticuerpos anti-PGL-I a títulos elevados. También se estudió la resistencia de las cepas a dapsona y rifampina. Cuatro de 18 muestras positivas por la reacción en cadena de la DNA polimerasa (PCR) tuvieron una mutación en el gene *fol*P, y una de 10 muestras positivas por PCR tuvo una mutación en el gene *rpo*B.

Estos resultados indican que el análisis serológico de los pacientes puede ser útil para detectar a los individuos de alto riesgo, y que en Pakistán hay cepas resistentes a la quimioterapia.

TO THE EDITOR:

In Pakistan, the multi-drug therapy (MDT) program against leprosy conducted by the World Health Organization (WHO) to eliminate the disease was quite successful, and the present prevalence rate is 0.1 per 10,000 inhabitants. However, there are "hot spot areas" where the prevalence rates are still as high as 3.4 per 10,000. Although a significant reduction of the total number of cases registered was observed, no apparent reduction of new cases was achieved ([9]), and the WHO has now recognized a necessity of a serious concern for leprosy control. One of the ways to achieve disease elimination is an active epidemiological surveillance of patient contacts in highly endemic "hot spot areas," which will be directly associated with detection of leprosy patients at an early stage.

On the other hand, although MDT was designed to prevent the emergence and spread of drug resistant strains, resistant *Mycobacterium leprae* strain have emerged. A strain showing resistance to both dapsone and rifampin was reported in 1993 ([3]) and, at present, there are even reports indicating the emergence of a strain resistant to multiple drugs ([6]). These drug resistant strains provide another serious problem and should not be ignored, especially in countries where the leprosy elimination goal has been achieved. Therefore, the development of a useful tool for early detection of leprosy and drug resistant strains is necessary for the prompt initiation of better medication.

In this study, we conducted serological surveillance of household and occupational contacts, and detected drug resistant strains in Karachi, a representative endemic area in Pakistan.

Serological test for leprosy. A total of 300 sera from various individuals, including in-and-out patient of CDGK Leprosy hospital, were obtained with informed consent. These sera were donated by 34 leprosy patients under treatment, 193 household contacts, 59 occupational contacts, and 14 non-contact healthy individuals living in Karachi (Table 1). Infection with *M. leprae* was assessed by using SERODIA®-leprae kit (Fuji Rebio Inc., Tokyo, Japan), which detects antibody against phenolic glycolipid-I (PGL-I) ([1]). Four leprosy patients under treatment were still found to be anti-PGL-I antibody positive (Table 1), and they were

TABLE 1. *Detection of anti-PGL-I antibody in sera from leprosy patients and their contacts.*[a]

Group	No.of sera examined	No.of positive sera	Percent positivity	No. of positive sera at each serum dilution				
				1:32	1:64	1:128	1:256	1:>512
Lepromatous leprosy patients	20	4	20	0	2	0	0	2
Borderline leprosy patients	8	0	0	0	0	0	0	0
Tuberculoid leprosy patients	6	0	0	0	0	0	0	0
Household contacts (children)	61	7	11.5	0	3	0	3	1
Household contacts (adults)	132	9	6.8	4	2	1	2	0
Occupational contacts	59	14	23.7	2	5	3	2	2
Non contacts	14	3	21.4	0	1	1	1	0
Total	300	37	12.3	6	13	5	8	5

[a]Detection of anti-PGL-I antibodies in serially diluted sera by ELISA using NT-P-BSA antigen coated gelatin particles.

Serum dilution of more than 1:32 showing agglutination was taken as positive.

TABLE 2. *Detection of drug resistant associated gene mutations of clinical isolates of* M. leprae.*

Place	No. of samples	*fol*P gene		*rpo*B gene	
		No. amplified†	Mutation	No. amplified	Mutation
Karachi	24	8	1	5	1
Peshawar	5	5	1	5	0
Balakot	10	5	2	0	0
Total	39	18	4	10	1

*Drug resistance related-genes, *fol*P and *rpo*B were amplified by PCR, sequenced, and compared with control *M. leprae* strain, Thai 53.

†Number of samples successfully amplified by PCR.

all lepromatous leprosy patients. However, borderline or tuberculoid leprosy patients had no antibodies against PGL-I. We then examined 193 household and 59 occupational contacts. Among household contacts, 11.5% of children had the antibody as did 6.8% of adult contacts (Table 1). Furthermore, 23.7% of occupational contacts had the antibody. Three out of 14 non-contacts were antibody positive. Further studies should be conducted with a larger number of non-contacts, but presently, we could not obtain informed consent from them. The titers among child contacts and occupational contacts are surprisingly high, which may indicate that some individuals were exposed to *M. leprae*. This is in accordance with a report that the seroprevalence rate was 26 to 28% in the high endemic area, and 7% in the low endemic area in Sulawesi, Indonesia ([7]). When we measured the antibody in a semi-quantitative fashion, individuals having high antibody titer were found in household and occupational contacts. The titers of antibody varied from low (1:32) to high (1:>512) values. Three cases out of 252 (1.2%) samples showed quite high (1:>512) antibody titer. These individuals should have a clinical examination to monitor the leprosy manifestation. It has been reported that anti-PGL-I antibody level can reflect the disease activity ([2]). Therefore, it might be reasonable to speculate that the antibody production was suppressed by successful MDT treatment.

Detection of drug resistant *Mycobacterium leprae*. Multi-bacillary (MB) type leprosy patients, either under or after MDT treatment, were targeted to obtain bacilli in the biopsy specimen. *M. leprae* genomic DNA was extracted from the specimens as described previously ([5]).

To detect drug resistant *M. leprae*, based on the previous studies ([4, 6, 8]), we targeted mutations of the *fol*P gene encoding dihydropteroate synthase (DHPS) for dapsone ([5]), and the *rpo*B gene for rifampin resistance ([4, 8]). The polymerase chain reaction (PCR) conditions and primers for *fol*P and *rpo*B are as described previously ([5, 6]). The amplified products from each primer pair were sequenced by using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Norwalk, CT, U.S.A.).

Thirty-nine skin samples were taken from leprosy patients in endemic areas of Pakistan such as Karachi, Peshawar, and Balakot, to detect gene mutations relating to drug resistance (Table 2). The number of samples successfully amplified using primers for *fol*P gene from 39 biopsy specimens was 18. Among amplified samples, four samples showed *fol*P mutations (22.2%). The *fol*P gene mutations were found at position 158th (the numbering system following that of reference 5) in three samples, and position 164th in one sample. These mutations induce amino acid changes from threonine to isoleucine at position 53rd of DHPS and from proline to arginine at 55th, respectively (not shown). These mutations have most commonly been observed in dapsone resistant strains ([5]). Although a larger number of samples should be analyzed, these observations may indicate that there are dapsone-resistant *M. leprae* in Pakistan. In contrast to *fol*P gene, primer pair for *rpo*B less frequently amplified the DNA. The possible reason for the failure might be the presence of less than detectable level of *M. leprae* bacilli. In our hands, the detection limit is approximately ten bacilli per biopsy sample. Also the different amplification efficiency between *fol*P

and *rpo*B might depend on a difference of the specificity of primers for each gene. Among ten *rpo*B gene samples amplified from the 39 biopsies, one sample showed the gene mutation at position 550th of the *M. leprae* ß subunit gene of RNA polymerase. This position was not a so-called "hot spot" of *rpo*B-associated resistant mutations ([8]); however, it induced a change of amino acid residue from aspartic acid to glycine (not shown). There was no relationship among the resistant samples, and no double mutation encoding both *fol*P and *rpo*B genes was observed.

It is not easy to determine whether the resistant strain developed before or after introduction of MDT. However, there might be some patients who are inadequately treated with MDT due to economical or other social reasons. These patients have a higher risk to produce mutidrug-resistant strain than patients adequately treated. Active surveillance is required for control of the spread of drug resistant *M. leprae*.

Taken together, we showed that some leprosy patient contacts have been infected with *M. leprae*. Also, dapsone resistance has been detected in Pakistan.

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

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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 bacteriocidal actions, including host immune system and antimycobacterial 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

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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.